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Franz-Xaver Reichl
Michael Schwenk
Editors

Regulatory Toxicology

Second Edition

Springer

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With 236 Figures and 127 Tables

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Editors
Franz-Xaver Reichl
Department of Operative/Restorative Dentistry
Periodontology and Pedodontics
University Hospital, LMU
München, Germany

Walther-Straub-Institute of Pharmacology and Toxicology
LMU Munich
Munich, Germany

Michael Schwenk
Formerly: Hannover Medical School
Hannover, Germany

Tübingen, Germany

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Foreword of the First Edition

Regulatory toxicology requires knowledge of aspects related to a number of disciplines of natural and social sciences, including chemistry, biology, bioinformatics, toxicology, epidemiology, exposure assessment and nutrition, as well as sociology, psychology and communication, to name a few. It builds a bridge between science and decision-making. In fact, risk analysis, the centrepiece of regulatory toxicology, is often seen as an art, in addition to its being a science. This explains why standard textbooks on regulatory toxicology are rare.

Ten years after the publication of the first standard work on regulatory toxicology in German, an updated, expanded version is now at hand in English language. This major publication addresses questions covering various aspects of risk assessment and risk management in general, paying attention to a number of fields including health protection, occupational health, environmental health and consumer protection. Basic principles are outlined, new developments described, and scientific, social and philosophical questions discussed at length. In times of an increased understanding that risk assessment and management need to be conducted in full transparency and with full involvement of all stakeholders, issues of risk perception and risk communication are addressed.

This breadth of information, complemented by a listing of the newest guidance values, makes this book a standard reference to those involved in the field of regulatory toxicology. It is of interest to risk scientists of various backgrounds, to policymakers and their advisors, and also to informed consumers.

A welcome and timely publication, indeed.

WHO, Geneva, Switzerland — Maged Younes
IUTOX-president, Aarhus, Denmark — Herman Autrup

Preface

Based on the success of the first edition (2014) of the book *Regulatory Toxicology*, the editors decided to compile a second edition. Though the basics of toxicological risk assessment and risk management experienced only moderate changes, the methods to study and evaluate adverse chemico-biological interactions were further refined in the years since the first edition, not least due to numerous novel in vitro test systems. Moreover, we experienced a decade of many advances in the consolidation of rules and guidelines on national and international levels.

The chapters of the first edition were updated for this second edition. Moreover, many new chapters were included, dealing with novel developments, such as regulations of nanoparticles, designer drugs, tattoos, biopharmaceuticals and gene therapy. The chapters were written by experts for all those who are involved in toxicological data generation and decision-making in institutions of pure and applied regulatory toxicology.

In summary, this handbook deals comprehensively with the management of chemical hazards and risks in regulatory toxicology, with a focus on test systems and their interpretation, and the concepts used to estimate acute and chronic toxicity to humans and the environment. Such information supports the work of those involved in risk assessment in academia, industry, government or related agencies. It also serves the superior aim of contributing to the protection of human health, clean environments, and a sustainable development on earth.

The editors have sought to bring consistency to the diversity of approaches and of opinions concerning toxicological risk assessment. Guideline values, regulations and data may change with time and among jurisdictions. However the basic principles and approaches discussed in this handbook will continue to provide a stable basis for future decision finding. Opinions expressed are those of the individual authors and do not necessarily represent the views of their institutions.

September 2021

Franz-Xaver Reichl
Michael Schwenk

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About the Editors

Franz-Xaver Reichl
Department of Operative/Restorative Dentistry
Periodontology and Pedodontics
LMU, Munich, Germany

Walther-Straub-Institute of Pharmacology
and Toxicology
LMU, Munich, Germany

Franz-Xaver Reichl studied microbiology and medicine in Munich, Germany. He then qualified as a toxicologist/pharmacologist and works at the Ludwig Maximilians University of Munich (LMU) as a dental-toxicologist at the Dental Clinic and at the Walther-Straub-Institute of Pharmacology and Toxicology at the LMU. His research is mainly related to molecular, cellular, and analytical (dental) toxicology and toxicity. F.X. Reichl consulted health agencies in toxicological risk management and is member of toxicological/pharmacological committees. He is head of the "International Advisory Board for the Biocompatibility/Toxicology of Dental Materials" at the LMU and is "Officer for Biosafety of Bacteria and Viruses" at the LMU Munich.

Michael Schwenk
Medizinische Hochschule Hannover
Center of Pharmacology and Toxicology
Hannover, Germany

Michael Schwenk studied biochemistry and medicine in Tübingen, Germany, and thereafter specialized in pharmacology, toxicology, and nutritional and environmental medicine. He was appointed professor in Pharmacology and Toxicology in 1987. He worked in

universities, basic research institutions, pharmaceutical industry, and a state health agency.

He created and headed the Regulatory Toxicology Study Group of the German Society of Toxicology, consulted health agencies in toxicological risk management, and was secretary of the IUPAC Division Chemistry and Human Health.

His research areas include membrane transport of xenobiotics, drug-biomolecule interactions, drug metabolism, cytotoxicity, phase I clinical studies, human biomonitoring, and risk assessment.

Contributors

Vincenzo Abbate Department of Analytical, Environmental and Forensic Sciences, King's College London, Franklin-Wilkins Building, London, UK

Hans-Juergen Ahr Investigational Toxicology, Bayer AG, Wuppertal, Germany

Klaus E. Appel Federal Institute for Risk Assessment, Berlin, Germany

Walter Aulmann Ecolab Deutschland GmbH, Monheim, Germany

Kathrin Begemann Department Exposure, Unit Exposure Assessment of Hazardous Products, German Federal Institute for Risk Assessment, Berlin, Germany

A. Bitsch Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM), Hannover, Germany

Ingrid Boekhoff Walther Straub Institute of Pharmacology and Toxicology, Ludwig-Maximilians-Universität München, Munich, Germany

H. M. Bolt Leibniz Research Centre for Working Environment and Human Factors, Dortmund, Germany

Scott Boyer Synapse Managers, Barcelona, Spain

Albert Braeuning Department "Food Safety", German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Andreas Breit Walther Straub Institute of Pharmacology and Toxicology, Ludwig-Maximilians-Universität München, Munich, Germany

Pieter Brekelmans National Poisons Information Center (NVIC), University Medical Center Utrecht, GA, Utrecht, The Netherlands

Hermann Broll Department "Food Safety", German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Eva A. M. Büch Leipzig University Cancer Center (UCCL), University of Leipzig Medical Center, Leipzig, Germany

Thomas R. H. Büch Rudolf Boehm-Institute for Pharmacology and Toxicology, Clinical Pharmacology, Leipzig University, Leipzig, Germany

Hans-Joerg Burger Formerly: Merck & Co., Kenilworth, NJ, USA
Morristown, NJ, USA

Reinhold Burr General Practitioner Practice, Hirrlingen, Germany

Alexander Cartus Food Chemistry and Toxicology, University of Kaiserslautern, Kaiserslautern, Germany

M. A. Cassar Voisin Consulting Life Sciences, Lausanne, Switzerland

Carlo Chiavaroli Voisin Consulting Life Sciences, Lausanne, Switzerland

Rory B. Conolly Integrated Systems Toxicology Division, NHEERL/ORD, U.S. Environmental Protection Agency, Research Triangle Park, NC, USA

Otto Creutzenberg Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany

Ronald de Groot National Poisons Information Center (NVIC), University Medical Center Utrecht, GA, Utrecht, The Netherlands

Wolfgang Dekant Formerly: Institute for Toxicology, University of Würzburg, Würzburg, Germany

Herbert Desel Department Exposure, Unit 32, Exposure Assessment of Hazardous Products, German Federal Institute for Risk Assessment, Berlin, Germany

Clara Desvignes Voisin Consulting Life Sciences, Saint-Gregoire (Rennes), France

Hermann H. Dieter Department of Toxicology of Drinking-Water, Federal Environment Agency of Germany/Umweltbundesamt (UBA), Dessau-Roßlau, Germany

Mike DiPaolo BioTherapeutic Development and Supply, Janssen Research and Development, Malvern, PA, USA

Kevin Doughty Registration Department, Bayer CropScience, Monheim, Germany

Hans Drexler Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine, University of Erlangen-Nürnberg, FAU, Erlangen, Germany

John H. Duffus Toxicology, The Edinburgh Centre of Toxicology, Edinburgh, Scotland, UK

Jürgen Durner Department of Conservative Dentistry and Periodontology, University Hospital, Ludwig-Maximilians-University of Munich (LMU), Munich, Germany

Birgit Dusemund Department of Food Safety, Federal Institute for Risk Assessment (BfR), Berlin, Germany

Alexander Eckhardt Toxicology of Drinking Water and Swimming Pool Water, German Environment Agency, Bad Elster, Germany

Lutz Edler Dudenhofen, Germany

Formerly: Division of Biostatistics, German Cancer Research Center-DKFZ, Heidelberg, Germany

Wolfgang Eisenmenger Ethics Committee Ludwig-Maximilians-University Munich, Munich, Germany

Andreas Eisenreich Department "Food Safety", German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Heidrun Ellinger-Ziegelbauer Investigational Toxicology, Bayer AG, Wuppertal, Germany

Norbert Englert Formerly: Federal Environmental Agency, Berlin, Germany

S. E. Escher Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM), Hannover, Germany

Rainer Fehr Department of Public Health, University of Bielefeld, Bielefeld, Germany

Esther Feistkorn Exposure Assessment of Hazardous Products, German Federal Institute for Risk Assessment, Berlin, Germany

Johannes Georg Filser Institute of Toxicology and Environmental Hygiene, Technical University of Munich, Munich, Germany

Roland Frötschl Genetic and Reproductive Toxicology, Federal Institute for Drugs and Medical Devices, Bonn, Germany

Tamara S. Galloway College of Life and Environmental Sciences: Biosciences, University of Exeter, Exeter, UK

David W. Gaylor Formerly: National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR, USA

Nikolaos Georgiadis Toxicologist, European Chemicals Agency (ECHA), Helsinki, Finland

Michael Giulbudagian Department of Chemicals and Product Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Thomas Göen Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany

Helmut Greim Institute for Toxicology and Environmental Hygiene, Technical University Munich, Freising-Weihenstephan, Germany

Miriam Große Hovest Ecolab Deutschland GmbH, Monheim, Germany

Thomas Gudermann Walther Straub Institute of Pharmacology and Toxicology, Ludwig-Maximilians-Universität München, Munich, Germany

Claude Guillou European Commission – Directorate General Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Ispra, VA, Italy

Ursula Gundert-Remy Institute for Clinical Pharmacology and Toxicology/ Institut für Klinische Pharmakologie und Toxikologie, Charité Universitätsmedizin Berlin, Berlin, Germany

Rainer Gürtler Department of Food Safety, Federal Institute for Risk Assessment (BfR), Berlin, Germany

Stephen Harston Formerly: Head of GLP Quality Assurance, Hoechst AG (1977–1998), Hofheim am Taunus, Germany

Hans-Karl Heim Federal Institute for Drugs and Medical Devices, Bonn, Germany

Karl Georg Heimann Expert Regulatory Toxicology, Köln, Germany

Karin Heine FoBiG – Forschungs- und Beratungsinstitut Gefahrstoffe GmbH, Freiburg, Germany

Gerhard Heinemeyer Formerly German Federal Institute for Risk Assessment, Berlin, Germany

Jan G. Hengstler Leibniz Research Centre for Working Environment and Human Factors at the Technical University of Dortmund (IfADo), Dortmund, Germany

Beate Henrikus Ethics Committee Ludwig-Maximilians-University Munich, Munich, Germany

Dietrich Henschler Formerly: Institute for Toxicology, University of Würzburg, Würzburg, Germany

Rolf Hertel Formerly: Federal Institute for Risk Assessment, Berlin, Germany

Britta Hilgenberg Faculty of Textile and Clothing Technology, University of Applied Sciences Niederrhein, Moenchengladbach, Germany

Sebastian Hoffmann seh consulting + services, Paderborn, Germany

Ludwig A. Hothorn Leibniz University Hannover (retired), Hannover, Germany

Stephanie Hüser Department of Environmental Hygiene, Governmental Institute of Public Health of Lower Saxony, Hannover, Germany

H. Paul A. Illing Formerly: Centre for Occupational and Environmental Health, University of Manchester, Manchester, UK

Matt C. Jacobsen Regulatory Safety Centre of Excellence, Clinical Pharmacology and Safety Sciences, IMED Biotech Unit, Biopharmaceuticals R&D, AstraZeneca, Cambridge, UK

Simon Jäger Department of Clinical Pharmacology, University Hospital Tübingen, Tübingen, Germany

Andrea Kaifie Institute of Occupational, Social, and Environmental Medicine, Medical Faculty, RWTH Aachen University, Aachen, Germany

Bernd Kaina Department of Toxicology, University Medical Center, Mainz, Germany

Helena Kandarova Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine SAS, Slovak Academy of Sciences, Bratislava, Slovakia

Andreas D. Kappos Formerly: Department of Health and Environment, Hamburg Health Authority, Frankfurt, Germany

Wolfgang Kaufmann Merck KGaA, Non-Clinical Safety, Global Pathology and Reproduction Toxicology, Darmstadt, Germany

Kai Kehe Department F – CBRN Medical Defense, Military Medical Research and Development, Bundeswehr Medical Academy, Munich, Germany

Agnieszka Kinsner-Ovaskainen Institute for Health and Consumer Protection, European Commission DG Joint Research Centre, Varese, Ispra, Italy

Fanny Kling Voisin Consulting Life Sciences, Lausanne, Switzerland

Thomas Kraus Institute of Occupational, Social, and Environmental Medicine, Medical Faculty, RWTH Aachen University, Aachen, Germany

Alain Krick Sanofi/Research/DMPK/Metabolism, Chilly Mazarin, France

Christian Laforsch Animal Ecology I and BayCEER, University of Bayreuth, Bayreuth, Germany

Alfonso Lampen Federal Institute for Risk Assessment, Berlin, Germany

Peter Laux Department of Chemicals and Product Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Stefan Lechtenböhmer Wuppertal Institute for Climate, Environment, Energy, Wuppertal, Germany

Lund University, Lund, Sweden

Wibke Lembke Zurich, Switzerland

Davina Lemm Ecolab Deutschland GmbH, Monheim, Germany

Bernhard Liebl Bavarian Health and Food Safety Agency, Oberschleissheim, Germany

Ines Liebl Bavarian State Ministry of Health and Care, Munich, Germany

Julika Lietzow Department "Food Safety", German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Marianne Lilienblum Former: Faculty of Social and Behavioural Sciences, University of Amsterdam, Amsterdam, Netherlands

Werner Lilienblum Hemmingen, Germany

Formerly: Lower Saxony State Authority for Occupational Safety and the Environment, Hannover, Germany

Mathias Locher Locher Biotech Consulting GmbH, Kuessnacht am Rigi, Switzerland

Andreas Luch Department of Chemicals and Product Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Claudia Luckert Department "Food Safety", German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Roman W. Lutz Formerly: Seminar for Statistics, Swiss Federal Institute of Technology, Zürich, Switzerland

Werner K. Lutz Institute of Pharmacology and Toxicology, University of Würzburg, Würzburg, Germany

Inge Mangelsdorf Department Chemical Safety and Toxicology, Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany

Peter Mayer Federal Institute for Drugs and Medical Devices, Bonn, Germany

Robert L. Maynard Birmingham University, Birmingham, UK

Odile Mekel Division Healthy Settings, NRW Centre for Health, Bochum, Germany

Jakob Menz Department "Food Safety", German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Simona Mondellini Animal Ecology I and BayCEER, University of Bayreuth, Bayreuth, Germany

Volker Mostert extera, Langenfeld (Rhld.), Germany

Harald Mückter Walther Straub Institute of Pharmacology and Toxicology, Ludwig-Maximilians-Universität München, Munich, Germany

Ludwig Müller Formerly: State Health Administration, unit 44 (Pharmaceutics, Toxicology, Genetic Engineering), Bremen, Germany

Manfred Neuberger Center of Public Health, Medical University of Vienna, Vienna, Austria

Hans-Günter Neumann Department of Toxicology, University of Würzburg, Würzburg, Germany

Birgit Niemann Department "Food Safety", German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Dennis Nowak Institute and Clinic for Occupational, Social and Environmental Medicine, LMU University Hospital Munich, Munich, Germany

Wolfgang Obergassel Wuppertal Institute for Climate, Environment, Energy, Wuppertal, Germany

Franz Oesch Institute of Toxicology, Johannes Gutenberg-University, Mainz, Germany

Klaus Olejniczak Formerly Non-clinical Regulatory Consultant, Berlin, Germany

Haluk Özkaynak U.S. Environmental Protection Agency (U.S. EPA), Durham, NC, USA

Jürgen Pauluhn Formerly Experimental Toxicology, Bayer HealthCare, Wuppertal, Germany

Nathan Pechacek Ecolab Inc, Eagan, MN, USA

Stefan Rakete Institute and Clinic for Occupational, Social and Environmental Medicine, LMU University Hospital Munich, Munich, Germany

Anja F. R. M. Ramsperger Animal Ecology I and BayCEER, University of Bayreuth, Bayreuth, Germany

Ulrich Ranft IUF – Leibniz Research Institute for Environmental Medicine, Düsseldorf, Germany

Franz-Xaver Reichl Department of Conservative Dentistry and Periodontology, University Hospital, LMU Munich, Germany and Walther-Straub-Institute of Pharmacology and Toxicology, LMU Munich, Munich, Germany

Deborah Revaud Voisin Consulting Life Sciences, Boulogne, Paris, France

Jens Riedel Sanofi-Aventis Deutschland GmbH/DMPK/Metabolism & tMSI, Industriepark Höchst, Frankfurt am Main, Germany

Julia Riehm Bundeswehr Institute of Microbiology, Munich, Germany

Leonard Ritter School of Environmental Sciences, University of Guelph, Guelph, ON, Canada

Elke Roehrdanz Federal Institute for Drugs and Medical Devices, Bonn, Germany

Cécile F. Rousseau Voisin Consulting Life Sciences, Boulogne, Paris, France

Manfred Ruthsatz Nutrition+HealthCARE, Vevey, Switzerland

Emmanuelle Sabbah-Petrover Voisin Consulting Life Sciences, Boulogne, Paris, France

Bernd Schäfer Department "Food Safety", German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Gottfried Schmalz Department of Conservative Dentistry and Periodontology, University Hospital Regensburg, Regensburg, Germany

Department of Periodontology, University of Bern, Bern, Switzerland

Klaus Schneider Forschungs- und Beratungsinstitut Gefahrstoffe GmbH, Freiburg, Germany

Dieter Schrenk Food Chemistry and Toxicology, University of Kaiserslautern, Kaiserslautern, Germany

Michael Schümann Department of Health and Consumer Protection, Hamburg, Germany

Matthias Schwab Department of Clinical Pharmacology, University Hospital Tübingen, Tübingen, Germany

Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany

Michael Schwenk Tübingen, Germany

Formerly: Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

Anuradha Shukla Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine, University of Erlangen-Nürnberg, FAU, Erlangen, Germany

Horst Spielmann Institute of Pharmacy, Freie Universität Berlin, Berlin, Germany

Neill H. Stacey Southern Cross Pharma Pty Ltd, Malua Bay, NSW, Australia

Thomas Steger-Hartmann Head of Investigational Toxicology, Bayer AG, Pharmaceuticals, Berlin, Germany

Dirk Steinritz Department F – CBRN Medical Defense, Bundeswehr Medical Academy, Munich, Germany

Roland Suchenwirth Department of Environmental Hygiene, Governmental Institute of Public Health of Lower Saxony, Hannover, Germany

Horst Thiermann Bundeswehr Institute of Pharmacology and Toxicology, Munich, Germany

Adam D. Thomas Centre for the Research in Biosciences (CRIB), UWE Bristol, Bristol, UK

Hartwig Visser Centre for the Research in Biosciences (CRIB), UWE Bristol, Bristol, UK

Emmanuelle M. Voisin Voisin Consulting Life Sciences, Boulogne, Paris, France

Gerhard Volland Material Testing Institute (MPA)-Otto-Graf-Institute Pfaffenwaldring, Schwäbisch Gmünd, Germany

Eckhard von Keutz Pharmaceuticals, Translational Sciences, Bayer AG, Wuppertal, Germany

Lutz Vossebein Faculty of Textile and Clothing Technology, University of Applied Sciences Niederrhein, Moenchengladbach, Germany

Sandra Wagener Department of Chemicals and Product Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Thomas Wallenhorst Sanitized AG, Burgdorf, Switzerland

David C. Watts School of Medical Sciences and Photon Science Institute, University of Manchester, Manchester, UK

Otto Schott Institute of Materials Research, Friedrich-Schiller-University, Jena, Germany

Gregory A. Wellenius Department of Environmental Health, Boston University School of Public Health, Boston, MA, USA

Michael Werner Wöllstein, Germany

Petra Wiedemeier Department Chemical Safety and Toxicology, Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany

Michael Wilhelm Department of Hygiene, Social and Environmental Medicine, Ruhr-University Bochum, Bochum, Germany

Klaus-Michael Wollin Formerly: Public Health Agency of Lower Saxony, Hannover, Germany

Maged Younes Global Publich Health, Mies, Switzerland

Formerly: Food Safety and Zoonoses, formerly: World Health Organization (WHO), Mies, Genève, Switzerland

Alexandre Zenié Joint Research Centre, European Commission, Ispra, Italy

Thomas Zilker Formerly Department for Clinical, Toxicology, Medizinische Klinik, Klinikum rechts der Isar, Technical University of Munich, Munich, Germany

Lothar Zöller Bundeswehr Institute of Microbiology, Munich, Germany

Part I

The "Modus Operandi" of Regulatory Toxicology

Aims and Mission of Regulatory Toxicology

1

Helmut Greim

Contents

H. Greim (✉)
Institute for Toxicology and Environmental Hygiene, Technical University Munich, Freising-Weihenstephan, Germany
e-mail: helmut.greim@mytum.de

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Abstract

The aim of regulatory toxicology is to control production, use, and deposition of dangerous materials to prevent adverse effects on human health and the environment. This requires sufficient information on the hazardous properties of a chemical compound, their relevance to man, and of human and environmental exposure, which is a prerequisite for appropriate risk assessment and the decision whether regulatory consequences are warranted. The three elements of risk assessment of chemical substances are hazard identification, evaluation of dose–response relationship, and exposure assessment. The risk assessment process requires differentiation between reversible and irreversible effects, that is, identification of a "no observed adverse effect level" (NOAEL) and/or a "lowest observed adverse effect level" (LOAEL) for the most critical effect of the substance, or, in case of irreversibility, estimation of the risk at a certain exposure. Up to now, the risk of genotoxic carcinogens at a certain exposure concentration is estimated by linear extrapolation of the dose–response curve. Further, to set priorities the margin of exposure (MOE) or margin of safety (MOS) can be calculated by comparing the NOAEL with the human exposure. Additional information for the evaluation of the human relevance of experimental data are available from toxicokinetics or the mode and/or mechanism of action. In the evaluation of the existing data base, setting limit values like "acceptable daily intake" (ADI) or "derived no effect levels" (DNELs) or implementing risk management measures, the uncertainties of the database have to be described and taken into consideration. Another challenge is the evaluation and regulation of mixtures, which needs to take into account concentrations and mode of action of the individual components. The systems for classification of carcinogens used by various national and international institutions are summarized. The use of the so-called precautionary principle and of the "Threshold of Toxicological Concern" (TTC) concept for risk assessment purposes is explained. In addition, the regulations for specific chemical classes like drugs or pesticides are completed by a short description of the European Union Registration, Evaluation, Authorisation and Restriction of Chemicals (EU REACH) regulation for chemical substances.

Keywords

Dose–response · Exposure · Hazard · Risk assessment · Test systems · Toxicokinetics · Mode of action · Regulation · Carcinogens · Agencies · Effects · Organ toxicity

Introduction

Regulatory toxicology uses the information on hazard and risks of human and environmental exposure of agents for their regulation. This requires understanding of the relevant regulations in this area as well as a basic understanding of the principles of toxicology. The latter is of specific importance because regulations are based on precise

numbers like the ADI (acceptable daily intake) or cut-off levels for the labeling of hazardous chemicals, whereas toxicology as a biological and experimental discipline does not provide such precise data. A NOAEL (No Observed Adverse Effect Level) is a number obtained from animal experiments using different doses. Its preciseness depends on the number of animals used at each dose and the difference to the LOAEL (Lowest Observed Adverse Effect Level). Depending on the dose intervals it might be 2- or 10-fold. Thus, expert judgment is required to correctly interpret the reliability of the data on which the ADI value is based and to decide on the need of regulatory consequences if the ADI is exceeded. Moreover, as implied by REACH regulations the socioeconomic consequences including the availability of alternatives and their own hazardous properties need to be considered. Although there are guidelines and regulations for almost all aspects of risk assessment, risk management, and regulations, it is the quality of hazard identification and risk assessment for human and the environmental exposure that determines the regulatory consequences. Consequently toxicology has to provide sufficiently defendable data for hazard identification and risk assessment and provide information, which are applicable for regulations as decided by the risk manager. On the other side the risk managers have to understand uncertainties of the risk assessment process and that a slight exceedance of an ADI or the risk control ratio (RCR) of 1 does not per se pose a nontolerable risk.

Since toxicology is the basis of all regulations to protect human health, a profound understanding of toxicology is essential for regulatory toxicology (c.f. Greim and Snyder 2019).

When a substance produces toxic alterations at a low dose, it is said to have a high potency. The type and extent of adverse effect is associated with duration and route of exposure (oral, inhalative, dermal) and the toxicokinetic behavior. Susceptible persons or groups may react already at lower dose than the general population. To characterize the risk of a given or potential exposure the adverse effects of chemicals have to be understood, and by evaluating the dose response to identify at what exposure a chemical will produce adverse effects.

It is obvious from this that risk characterization comprises three elements:

- Hazard identification, that is, a description of the agent's toxic potential
- Evaluation of the dose–response, including information on the concentration above which the agent induces toxic effects to identify the NOAEL
- Exposure assessment to understand the concentration of the agent in the relevant medium, time, and routes of human exposure

Components of Risk Assessment

Hazard Identification

Chemicals induce local and/or systemic effects such as embryotoxicity, hepatotoxicity, and neurotoxicity after absorption from the gastrointestinal tract, through the skin or via the lungs.

Depending on exposure concentration and time of exposure acute or chronic effects may result. Acute intoxication usually occurs in response to large doses of the parent compound less due to its metabolites. Acids or bases are directly acting agents which cause local irritation or corrosion at the site of exposure, such as the nasal and occular surfaces, the respiratory tract, mouth, esophagus, or skin.

Highly lipophilic substances, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) accumulate in tissues especially in body fat because they are lipophilic and are not well metabolized. In humans the half-life of excretion of TCCD is about 8 years. To insure safe use by the consumers or the specific conditions in the workplace the toxicological profiles of each chemical need to be evaluated. Such evaluation may take different forms for new and existing chemicals. In the case of newly developed drugs, pesticides, or new chemicals a stepwise procedure is used starting from simple in vitro and in vivo short-term tests. Depending on the hazardous potential of the agent, studies are extended to evaluate long-term effects by repeated dose studies, toxicokinetics, and toxic mode of action. For existing chemicals the available information is collected and a risk assessment based on exposure data, knowledge of the dose-response relationship, and the mode of action can be performed or in case of data gaps additional experiments requested.

Reactivity, solubility, and metabolism of the chemical or its metabolites determines the target organ of the critical effects. Histopathological and biochemical changes have been the major parameters used to detect organ toxicity. Increasing availability of sensitive methods in analytical chemistry and molecular-biological approaches including toxicokinetics and the various "omics" has significantly improved the understanding of the mechanisms by which cellular and subcellular functions are impaired and how the cells are responding to toxic insults. This results in a better understanding of toxic mechanisms, species differences, and the consequences of exposures at high and low concentrations over different times.

Dose–Response and Toxic Potency

According to the general principle of toxicology as expressed by Paracelsus (1493–1541): "In all things there is a poison, and there is nothing without a poison. It depends only upon the dose whether a poison is poison or not" or in short "the dose makes the poison." The consequences of human or environmental exposures depend on the amount and duration to which these individuals or populations are exposed. This paradigm anticipated today's threshold concept which can be assessed in studies on dose–response relationships.

Animal or human exposure is usually defined as the dose, for example, in mg of the chemical/kg body weight/day. This daily dose may result from oral, inhalation, or dermal exposure or as a sum thereof. The external dose leads to a specific internal dose, which depends on the amount absorbed via the different routes. Absorption rates via the different routes can vary significantly, although oral and inhalation exposure usually lead to the highest internal dose. For example, about 50% of cadmium in tobacco smoke is absorbed in the lung, whereas cadmium absorption from the

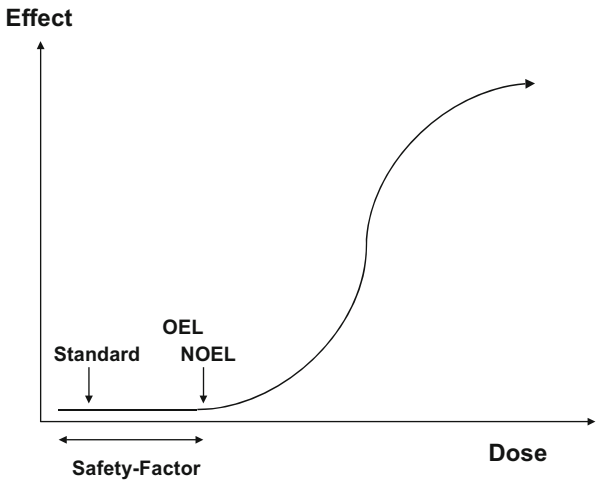

Fig. 1 Dose–response curve showing the dose on the X-axis and % response (Effect) on the Y-axis. The figure illustrates the location of regulatory values such as the NOEL (NOAEL), occupational exposure levels (OELs), or environmental standards such as acceptable daily intake (ADI). Note that a doubling of dose in the lower or upper part of the S-shaped curve results in small increases in effects, whereas they are much more prominent in the steep part

gastrointestinal tract is about 10%. Ultimately, it is the dose which reaches the cellular target over a given time period that results in the toxicological response. No toxic effects will be seen at doses up to the NOAEL, which is the starting point to derive values of acceptable exposures for consumers (acceptable daily intake (ADI)) or at the workplace (occupational exposure limits (OELs)). The dose–response curve may be expressed using a variety of mathematical formulas. Using the linear form of the dose–response relationship the curve is sigmoidal in shape and varies in slope from chemical to chemical. Thus, if the curve is shallow a doubling of the dose results in a small increase of effects, whereas effects increase severalfold when the slope is steep (see Fig. 1). The dose is plotted on the abscissa (X axis). The position of the sigmoidal part of the curve above the x-axis provides information about the toxic potency of a substance.

Exposure Assessment

According to the Paracelsian admonition exposure assessment or prediction of exposure is an ultimate requirement for risk assessment and to decide on the need for regulations.

Exposure defines the amount of a chemical to which a population or individuals are exposed via inhalation, oral, and dermal routes and is commonly defined by mg of the chemical/kg body weight per day.

Since occupational exposure is regular and repetitive it can easily be measured in the air of the workplace and/or by use of personal monitoring equipment; exposure of general population is more difficult to assess. It usually is a combination of the presence of the compounds in indoor/outdoor air, drinking water, food, or use of products that contain the chemical. Moreover, frequency, duration and site of exposure, concentration, and weight of substance in the products need to be

considered. Children represent a special case of exposure. They may be exposed to chemicals that are released from toys during mouthing, via skin contact or uptake of contaminated dust/soil by hand-to-mouth behavior. Exposure scenarios that estimate and quantify the frequency of contact, ingested amounts, solubilization, and the extent of absorption will help to model and roughly estimate the combined uptake from various sources.

Absorption through the skin will also influence the body burden of the chemical. Use of these parameters to assess exposure is plagued by many uncertainties, which often lead to overestimation of the actual exposure, because external exposure may not necessarily correlate with internal exposure. Biomonitoring of the compound or its reaction products in the exposed individuals provides the most reliable estimate of internal exposure. However, dose–response curves usually provide a correlation between external dose and effects. Therefore, risk assessment of an internal exposure either requires knowledge of the dose–response of internal exposure versus adverse effects or information to which extent external and internal doses correlate. The estimation of exposure is more complicated when mixtures of chemicals are the source of exposure.

Ultimately, it is the dose that reaches the cellular target over a given time period that results in the toxicological response. Thus, the toxic potency of a chemical is the product of the interrelated external, internal, and target doses, which results from the multiple pathways and routes of exposure to a single chemical (aggregate exposure). In the case of existing chemicals an appropriately designed program to measure the chemical in the different media will provide the necessary information.

The measurement of external dose is either done on collected samples, such as food samples or by direct measurement, that is, in air. When collected samples are used representative sampling and appropriate storage conditions as well as accurate and reproducible measurement techniques are essential. This also applies to biomonitoring programs.

Exposure data are not fully known for many chemicals, and exposure-modeling is therefore an important tool.

The Technical Guidance Document on Risk Assessment Part I (European Chemicals Agency (ECHA) 2003) of the European Commission lists the following core principles for human exposure assessment for new substances, existing substances, and biocides:

- Exposure assessments should be based upon sound scientific methodologies. The basis for conclusions and assumptions should be made clear and be supportable and any arguments developed in a transparent manner.
- The exposure assessment should describe the exposure scenarios of key populations undertaking defined activities. Such scenarios that are representative of the exposure of a particular (sub) population should, where possible, be described using both reasonable worst-case and typical exposures. The reasonable worst-case prediction should also consider upper estimates of the extreme use and reasonably foreseeable other uses. However, the exposure estimate should not be grossly exaggerated as a result of using maximum values that are correlated with each other. Exposure as a result of accidents or from abuse shall not be addressed.

- Actual exposure measurements, provided they are reliable and representative for the scenario under scrutiny, are preferred to estimates of exposure derived from either analogous data or from the use of exposure models.
- Exposure estimates should be developed by collecting all necessary information (including that obtained from analogous situations or from models); evaluating the information (in terms of its quality, reliability, etc.); thus enabling reasoned estimates of exposure to be derived. These estimates should preferably be supported by a description of any uncertainties relevant to the estimate.
- In carrying out the exposure assessment the risk reduction/control measures that are already in place should be taken into account. Consideration should be given to the possibility that, for one or more of the defined populations, risk reduction/control measures that are required or appropriate in one use scenario may not be required or appropriate in another (i.e., there might be subpopulations legitimately using different patterns of control which could lead to different exposure levels).

Exposure-biomonitoring provides direct information about the internal exposure of organs. It allows to measure or estimate:

- The aggregate exposure that reflects the quantity of a substance that has entered the organism via all routes.
- Its biotransformation and biological half-life and the estimated total body burden at the time of measurement.
- The exposure of target organ to the original substance and its metabolites.
- Exposure-biomonitoring is an important diagnostic tool that allows to estimate whether a chemical exposure via the air, food, or contaminated house dust causes increase of the internal body load, as compared to populations that are not specifically exposed.

Effect-biomonitoring is a diagnostic tool that investigates substance-induced changes of enzyme activities or other molecular and cellular parameters.

Risk Assessment

Advanced methods of sample preparation and chemical analysis are available that can detect extremely small substance levels in environmental media and biological tissues. When a chemical is found, this alone does not mean that it is accompanied with a toxic response of an exposed individual. According to the Paracelsus paradigm, dose is an essential parameter that determines toxic response. Only when the NOAEL is exceeded, an effect can be expected.

It is essential to differentiate between effects that are reversible and others that may be irreversible and associated with chronic disorders. Substances that induce reversible effects have a segment in the lower dose range of their dose–response plots, below which no adverse effect can be observed. The discrimination point is the NOAEL. The first dose that elicits an adverse effect is the LOAEL. When using the terms NOAEL and LOAEL, an analysis should be made, whether the observed

response is a physiological adaptation or an adverse effect. It is to be noted that threshold is not the equivalent of an NOAEL, since it describes a concentration or exposure where the slope of the dose–response curve changes.

If damage is not repaired and/or eliminated, the effect persists, may accumulate upon repeated exposure, and becomes irreversible. Reversibility depends on the regenerative and repair capacity of cells, subcellular structures, and macromolecules during and after exposure. Epithelial cells of the intestinal tract or the liver have a high regenerating capacity and rapidly replace damaged cells by increased cell replication. The highly specialized cells of the nervous system have lost this capacity during natal and post-natal development. Consequently damaged cells are not or slowly replaced, at least in the adult.

For chemicals that induce reversible effects the NOAEL of the most sensitive endpoint is determined and compared with the human exposure to describe the margin of exposure (MOE) (or margin of safety (MOS)). If the NOAEL is derived from animal experiments, an MOE of 100 or greater is desirable, which comprises a factor of 10 for interspecies differences and another factor 10 for intraspecies differences. An MOE of at least 10 is sufficient if the NOEL is derived from human data.

The covalent binding of genotoxic mutagens and carcinogens to DNA is considered an irreversible event despite the availability of repair processes. However, there is increasing knowledge about DNA-repair mechanisms, the role of tumor-suppressor genes, apoptosis, and the level of background mutation rates, so that the assumption that even genotoxic effects exhibit a threshold becomes increasingly plausible (see Greim and Albertini 2015). So far the general agreement remains that the potency of genotoxic carcinogens increases with increasing dose without a NOAEL.

To determine the potency of genotoxic carcinogens and cancer risk at a given exposure, a linear or sublinear extrapolation from the high dose effects observed in animals to the usually lower human exposure is requested by regulatory agencies. The European Food and Safety Authority (EFSA) recommends to avoid this extrapolation because of the inherent uncertainties. Instead, the MOE between a benchmark dose, or the T25 calculated from a carcinogenicity study in animals and human exposure, should be determined. An MOE of 10,000 and more is of minor concern. The advantage is that neither a debatable extrapolation from high to low doses needs to be performed nor are hypothetical cancer cases calculated.

Requirements for Toxicological Hazard Identification and Risk Assessment

The identification of hazardous properties of a chemical includes the following studies:

- Acute, subchronic, and chronic toxicity (oral, inhalation, dermal)
- Genotoxicity (in vitro and in vivo methods)
- Carcinogenicity (lifetime studies)
- Irritation (skin, mucous membranes, eye) and phototoxicity

- Sensitization and photosensitization
- Reproductive and developmental toxicity
- Toxicokinetics
- Mode and mechanism of action
- Other aspects such as neurotoxicity and immunotoxicity

Wherever adequate, dose–response relationships, presence of thresholds, NOAEL, LOAEL, and maximal tolerated dose (MTD) should be studied. Guidelines are available for many test systems and assays (Organization for Economic Cooperation and Development (OECD) Guidelines). For existing chemicals a broad database is generally available and published by various organizations (see Table 1). The test systems have been continuously refined and improved in the past decades and it can be expected that additional novel test procedures will be developed in the future (c.f. Greim and Snyder 2019).

Acute Toxicity, Subchronic Toxicity, and Chronic Toxicity

Acute toxicity studies are performed in rodents. They employ a range of doses including the estimated lethal dose. Acute and delayed effects are observed during 2 weeks, and the acutely lethal dose is determined.

Table 1 International institutions that publish documentations on chemicals

ACGIH (American Conference of Governmental Industrial Hygienists) http://www.acgih.org/TLV/
ATSDR (The Agency for Toxic Substances and Disease Registry) http://www.atsdr.cdc.gov/
European Chemicals Agency echa.europa.eu
The Canadian Centre for Occupational Health and Safety http://www.ccohs.ca/
Dutch Expert Committee on Occupational Standards (DECOS), http://www.gr.nl/adviezen.php?Jaar=2012
Environmental Protection Agency (EPA) http://www.epa.gov/
European Centre for Ecotoxicology and Toxicology of Chemicals http://www.ecetoc.org/
UK Health and Safety Executive (HSE) http://www.hse.gov.uk/
International Agency for the Research of Cancer http://monographs.iarc.fr/ENG/Classification/index.php
International Programme on Chemical Safety http://www.inchem.org/
The Japanese Association of Industrial Health http://joh.med.uoeh-u.ac.jp
MAK Commission (German Research Foundation) https://onlinelibrary.wiley.com/doi/book/10.1002/3527600418
NIOSH http://www.cdc.gov/niosh/homepage.html
The Nordic Expert Group http://www.nordicexpertgroup.org//
OSHA http://www.osha.gov/
SCOEL – EC Scientific Committee on occupational exposure limits http://ec.europa.eu/employment_social/health_safety/docs_en.htm

In **repeated dose (subacute) studies** the chemical is administered to rodents daily for several weeks or months. In special cases larger species such as dogs, and in the case of new drugs, monkeys or apes may be employed. Possible toxic effects, for example, with regard to animal behavior, body weight, or common diagnostic parameters, are documented. At the end, the animals are sacrificed to examine organs for pathological and histopathologic alterations.

Chronic studies are characterized by repeated dosing of animals, in general rodents, for months up to a lifetime. They investigate possible effects of long-term exposure. A major goal of lifetime studies is the detection of possible carcinogenic effects.

There is an ongoing tendency to replace animal studies by in vitro studies and in silico prediction of structure–activity relationships. They are novel developments and contribute to reduction of cost as well as animal protection. At present, the information, deduced from these types of replacement studies, is restricted to selected endpoints. They cannot reflect the complex network and interactions of organs, hormones, transmitters, neurons, etc. and so it is presently not possible to fully replace in vivo methods.

Nevertheless, some in vitro systems can be used to estimate aspects of dose–response relationship and may contribute to toxicokinetic modeling and prediction of dose-dependent effects in the specific target. Test systems with a drug-metabolizing function may detect relevant biotransformation products and reactive intermediates. Nevertheless, this does not necessarily reflect the situation in the living organism. In fact, assessment of all relevant endpoints, their dose dependency, thresholds, and NOAELs can only be finally assessed in the intact animal. When such information is unavailable hazard identification is incomplete. Consequently the basis for appropriate risk assessment of human exposure is missing.

Genotoxicity

Substance-induced damage of DNA is usually detected by bacterial mutagenicity assays in the absence and presence of drug metabolizing enzymes as well as by an in vitro cytogenetic assay. This is supplemented by the mouse bone marrow micronucleus test that detects chromosomal abnormalities. When these tests show genotoxic potential a chemical substance is usually not further developed, unless for specific applications (e.g., chemotherapeutics). When there is unclear evidence for genotoxicity, carcinogenicity studies in the further characterization of the substance will provide further information.

Carcinogenicity

Carcinogenicity studies have a similar design as studies on chronic toxicity. Three or more doses in the range of the maximum tolerated dose are administered to animals (50 animals per sex and dose) with the aim to detect possible increases over the natural tumor incidence. Rats and mice have several advantages for such studies:

They have a lifespan of only 2 years, during which they develop from young to aged animals. There is plenty information about their physiology, pathology, and susceptibility to chemical tumor induction and promotion. Databases on spontaneous tumor incidences for many strains and their tissues exist. The incidence of spontaneous and substance-induced tumors tends to increase in older animals.

When interpreting results of tumor incidence, it must be considered that non-carcinogenic chronic toxic effects may have influenced the study outcome. For example, very high doses may result in insufficient detoxification of the test substance, decreased intracellular GSH levels, or overwhelmed DNA repair. Some forms of tumors such as the α-urinary globulin-induced kidney tumors of the male rat are species-specific. Their occurrence will hardly be relevant for humans. Also rodents are more sensitive to peroxisome proliferators. Results of carcinogenicity studies must be evaluated by experienced experts. They will take into account their experience about the pattern of toxic changes and possible underlying mechanisms to identify a substance as a carcinogen.

Irritation and Phototoxicity

Dermal irritation of substances is studied in animals and humans. It is common to use a single occluded patch. Substances having significant light absorption in the ultraviolet range (290–400 nm) carry a risk of phototoxicity and photoallergic reactions. Here, a substance-treated patch site is irradiated either immediately or after patch removal. In vitro assays for phototoxicity include the test for uptake of Neutral Red by 3 T3 cells. In the case of negative results, further testing in the living organism may not be necessary.

Sensitization

To study immunological sensitization, the Buehler skin test in guinea pigs and the local lymph node assay (LLNA) in mice are performed in animals, but novel tests such as human skin cultures are in development. The LLNA has been reported to correlate closely with the results gained by repeat patch testing in humans. In cases, where animal data indicate a potential for contact sensitization, human skin sensitizing testing is conducted, usually by a human repeated-insult patch test (HRIPT). If possible, the formation of specific antibodies in the course of substance exposure should be monitored.

Toxicity for Reproduction and Development

Such studies aim at detecting substance-induced changes of reproductive function of the parents, fetotoxic, teratological, or development-disturbing effects on the fetus and possible effects on the second generation.

Toxicokinetics

A chemical may enter the body via the gastrointestinal tract, the lung, or the skin. The amount absorbed depends on the concentration in the different media like food, air, on physical-chemical parameters such as solubility in water and fat, stability, and the route of exposure. Toxicokinetics describe absorption, distribution, metabolism and elimination (ADME) of a chemical in humans, or experimental animals. Of specific importance for interpretation of animal studies and for extrapolation between species is the comparative information on the exposure and the dose that reaches the critical target.

Upon inhalation or skin penetration the compound directly enters the cardiovascular system and distributes into the organs. When absorbed from the gastrointestinal tract the chemical enters the liver via the portal vein and to a much less extent is taken up directly into the cardiovascular system. The epithelial cells of the gut wall and the liver present a large capacity for metabolizing chemicals so that a compound may be extensively metabolized by this **"first pass effect"** before entering the (cardiovascular) systemic circulation. Larger molecules, for example, the glucuronosyl-conjugates can be excreted via the biliary system into the duodenum where the conjugates may be hydrolyzed so that the original compound is reabsorbed and reenters the liver. This process is defined as **enterohepatic circulation**. Inhalation or dermal exposure to a chemical, intravenous, or intraperitoneal injection may result in different effects than after oral exposure because of the "first pass effect."

After entering the cardiovascular system the chemical or its metabolites distribute to the organs where they can accumulate in organs such as fat or bones, or are further metabolized. Reactive metabolites will interact with tissue components and may induce cellular damage. This "tissue dose," that is, the concentration of a chemical or its metabolite at the critical target over a given time, is an important factor that helps to understand the correlation between internal exposure and external (environmental) exposure in relation to toxicity. By comparing tissue doses in different species at similar exposures also helps to understand species differences in the sensitivity to chemicals, as well as interindividual variations.

The chemical or its more water-soluble metabolites are primarily excreted via the kidneys or the biliary system. Volatile compounds may be exhaled. The great variety of processes observed during absorption, metabolism, distribution, and excretion cannot be predicted by modeling or by in vitro experiments without confirmatory data from animals and man.

Mode and/or Mechanism of Action

There are many mechanisms by which chemicals or other stressors like heat or radiation can lead to toxicity. Knowledge of the modes or mechanisms by which a chemical induces toxicity are essential to understand species specificities, species differences, sensitive populations, or the interpretation of data regarding threshold or non-threshold effects. They also help to evaluate the relevance of the toxic effects to

humans when the data are derived from experimental animals. Whereas toxic mechanism is often not known in detail, modes of action, which can be described in a less restrictive manner, are helpful in the risk assessment process as well. Generally toxic mechanisms or modes of action are differentiated as follows:

Physiological changes are modifications to the physiology and/or response of cells, tissues, and organs. These include mitogenesis, compensatory cell division, escape from apoptosis and/or senescence, inflammation, hyperplasia, metaplasia and/or preneoplasia, angiogenesis, alterations in cellular adhesion, changes in steroidal estrogens and/or androgens, and changes in immune surveillance.

Functional changes include alterations in cellular signaling pathways that manage critical cellular processes such as modified activities for enzymes involved in the metabolism of chemicals such as dose-dependent alterations in phase I and phase II enzyme activities, depletion of cofactors and their regenerative capacity, alterations in the expression of genes that regulate key functions of the cell, for example, DNA repair, cell cycle progression, post-translational modifications of proteins, regulatory factors that determine rate of apoptosis, secretion of factors related to the stimulation of DNA replication and transcription or gap–junction-mediated intercellular communication.

Molecular changes include reversibility or irreversibility of changes in cellular structures at the molecular level, including genotoxicity. These may be formation of DNA adducts and DNA strand breaks, mutations in genes, chromosomal aberrations, aneuploidy, and changes in DNA methylation patterns.

Mechanistic information is most relevant for the evaluation, classification, and regulation of all hazardous and at a given exposure to risky chemicals including carcinogens. For example, if the carcinogenic effect is induced by a specific mechanism that does not involve direct genotoxicity, such as hormonal deregulation, immune suppression, cytotoxicity, the detailed search for the underlying mode of action may allow identification of a NOAEL. This is also applicable for inert materials, such as poorly soluble fibers, dusts, and particles, which induce persistent inflammatory reactions as a result of their long-term physical presence that ultimately lead to cancer.

Evaluation of Uncertainties

When discussing regulations of specific chemicals, one has to be aware that the data used to set a DNEL or to determine the carcinogenic risk of the exposure to a certain chemical always include uncertainties, with the consequence that the risk of a certain exposure may be over- or underestimated. For example, the NOAEL may not be a real NOAEL for statistical reasons in that too few animals have been used in the specific experiment. Or the NOAEL is rather conservative because the next higher dose, which determines the LOAEL of a weak adverse effect, is 10-fold higher. Usually this uncertainty is covered by deliberately applying assessment factors that build in a margin of error so as to be protective of the population from risks. In case

of DNELs the uncertainty factor of 100 covers the uncertainties of inter- and intraindividual differences unless toxicodynamic and/or toxicokinetic information allows its reduction. Whereas the experts who have performed the risk assessment are usually aware of uncertainties, the risk manager tends to use the numbers as such, with the consequence that any exposure even slightly higher than the DNEL is not considered to be acceptable.

Acknowledging these uncertainties with data the distribution of uncertainties may be defined by statistical approaches to characterize and weight the different assumptions from various components (including dose–response, emissions, concentrations, exposure, valuation). This will improve understanding of the reliability of the available information, how risk may vary in a population, and thereby allowing better mean estimates of risk and of the magnitude of risk for different individuals.

There is an ongoing discussion about the risk of chemicals in toys, which can be mouthed by children. Obviously, the resulting exposure is determined by the migration rates of the chemical from the material and the daily mouthing time. Since there is no standard procedure to determine migration rates and the data on mouthing time published in the scientific literature differ by a factor of almost 10, the database for regulatory consequences is rather uncertain. This is why the Scientific Committee on Toxicology, Ecotoxicology and the Environment in its opinion on diethyl-hexylphthalate in teethers only concluded that the resulting exposure is only about 20-fold below the NOAEL instead of the usually applied factor of 100. Based on this information the European Commission restricted the use of diethyl-hexylphthalate in teethers **and** in all toys which can be mouthed by children. With this the Commission applied the precautionary principle, because such toys usually contain lower concentrations of diethyl-hexylphthalate and mouthing times will be less than for teethers.

Methods for the evaluation of unquantified uncertainties are described by EFSA (2006) and ECHA (2003).

Evaluation of Mixtures

Humans and their environments are exposed to a wide variety of substances. The potential adverse effects of the interactions between those substances when present simultaneously in a mixture have been analyzed in several reviews and documentations. Most recently the available scientific literature has been analyzed by the nonfood scientific committees of the European Commission. The following conclusion have been reached (see SCCS/SCHER/SCENIHR (2012a)):

1. Under certain conditions, chemicals will act jointly in a way that the overall level of toxicity is affected.
2. Chemicals with common modes of action will act jointly to produce combination effects that are larger than the effects of each mixture component applied singly. These effects can be described by dose/concentration addition.
3. For chemicals with different modes of action (independently acting), no robust evidence is available that exposure to a mixture of such substances is of health or

environmental concern if the individual chemicals are present at or below their zero effect levels.
4. Interactions (including antagonism, potentiation, and synergies) usually occur at medium or high dose levels (relative to the lowest effect levels). At low exposure levels, they are either unlikely to occur or are toxicologically insignificant.
5. In view of the almost infinite number of possible combinations of chemicals to which humans and environmental species are exposed, some form of initial filter to allow a focus on mixtures of potential concern is necessary. Several criteria for such screening are offered.
6. With regard to the assessment of chemical mixtures, a major knowledge gap at the present time is the lack of exposure information and the rather limited number of chemicals for which there is sufficient information on their mode of action. Currently, there is neither an agreed inventory of mode of actions nor a defined set of criteria how to characterize or predict a mode of action for data-poor chemicals.
7. If no mode of action information is available, the dose/concentration addition method should be preferred over the independent action approach. Prediction of possible interaction requires expert judgment and hence needs to be considered on a case-by-case basis.

Classification of Carcinogens

The systems for classification of carcinogens used by various national or international institutions were developed in the 1970s. Classification is based on qualitative criteria, and reflects essentially the weight of evidence available from animal studies and epidemiology. Classification is usually based on the certainty with which a carcinogenic potential for a chemical can be established. Generally three categories, the definitions of which slightly differ, are used.

- Human Carcinogens
- Animal Carcinogens, reasonably anticipated to be human carcinogens
- Not classifiable because of inadequate data

For classification, mode of action and potency of a compound are either not taken into account or at best is used as supporting arguments. The advancing knowledge of reaction mechanisms and the different potencies of carcinogens may lead to a reevaluation of the traditional concepts.

The International Agency for Research of Cancer (IARC) and the OECD propose to use data on the carcinogenic mechanism and potency in decision-making. The SCOEL (Scientific Committee for Occupational Exposure Limits, now part of ECHA) of the General Directorate Employment of the European Union applied information on carcinogenic mechanisms and potency as criteria for a revised classification. The US Environmental Protection Agency (EPA) and a committee of the German Research Foundation (Deutsche Forschungsgemeinschaft) recommended consideration of mode

of action and have published modified concepts for classification. These activities in part originate from the recognition that one can distinguish between mechanisms of carcinogenicity caused by non-genotoxic and genotoxic carcinogens. Thus, it is possible to identify a NOAEL for non-genotoxic carcinogens, provided there is sufficient information on the primarily non-genotoxic mechanism. The American Conference of Governmental Industrial Hygienists (ACGIH) uses a concept, which considers carcinogenic potency for classification since 1995.

As indicated above the REACH uses the C&L criteria of the Globally Harmonised System (GHS), which is exclusively hazard based. This leads to classification of CMR (carcinogenic, mutagenic, reproductive) compounds without considering whether the test conditions of the animal experiments are relevant for humans or whether there is human exposure which may result in a risk. Since classification in CMR Categories 1A or 1B has consequences for consumer exposure, this may lead to scientifically non-justified restrictions. Although industry can submit a proposal for authorization the severe consequences of a toxicologically and socioeconomically not justified C&L can be avoided if the C&L process would become risk based.

The Precautionary Principle

According to Article 191 of the Treaty on the Functioning of the European Union (EU) the precautionary principle aims to assure a higher level of environmental protection through preventative decision-taking in the case of risk. However, in practice, the scope of this principle is far wider and also covers consumer policy, European legislation concerning food and human, animal, and plant health. It is a measure to enable rapid response in the case of a possible danger to human, animal, or plant health, or to protect the environment. In particular, where scientific data do not permit a complete evaluation of the risk, this principle may, for example, be used to stop distribution or order withdrawal from the market of products likely to be hazardous.

Since this description allows various interpretations, a more precise definition is given in the Communication from the Commission of February 2, 2000, on the precautionary principle. There it is outlined that the precautionary principle may be invoked when a phenomenon, product, or process may have a dangerous effect, identified by a scientific and objective evaluation, if this evaluation does not allow the risk to be determined with sufficient certainty. The Commission specifically stresses that the precautionary principle may only be invoked in the event of a potential risk and that it can never justify arbitrary decisions. To allow its application three preliminary conditions should be met:

- The fullest possible scientific evaluation, the determination, as far as possible, of the degree of scientific uncertainty
- A risk evaluation and an evaluation of the potential consequences of inaction
- The participation of all interested parties in the study of precautionary measures, once the results of the scientific evaluation and/or the risk evaluation are available

In addition, the following general principles of risk management remain applicable when the precautionary principle is invoked:

- Proportionality between the measures taken and the chosen level of protection
- Nondiscrimination in application of the measures
- Consistency of the measures with similar measures already taken in similar situations or using similar approaches
- Examination of the benefits and costs of action or lack of action
- Review of the measures in the light of scientific developments

Other states use slightly different definitions. For example, the Canada definition is as follows:

> The precautionary principle is an approach to risk management that has been developed in circumstances of scientific uncertainty, reflecting the need to take prudent action in the face of potentially serious risk without having to await the completion of further scientific research.

Canada refers to the definition of the Precautionary Principle of the Rio Conference on Environment and Development (Principle #15 of the June 1992, Declaration), which reads:

> In order to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation.

Although these definitions seem to be contradictive they generally indicate that the precautionary principle should be applied in cases of potentially *serious* risks without having to wait for a complete risk assessment. This implies that the principle is only applicable in case of a severe risk in case it cannot be sufficiently defined at present.

The TTC Concept

The Threshold of Toxicological Concern (TTC) is a concept to establish a level of exposure for chemicals, regardless their chemical-specific toxicity data, below which there is no appreciable risk to human health. The concept is based on knowledge of the chemical structure to evaluate structural alerts, the amount of a specific chemical in a product, and the daily human exposure. So far the TTC concept is applied for chemicals in food. It is defined as a nominal oral dose which poses no or negligible risk to human health after a daily lifetime exposure. At a mean dietary intake below the level of the TTC, toxicology safety testing is not necessary or warranted. By that the TTC concept can contribute to a reduction in the use of animals for safety tests. The TTC concept may also represent an appropriate tool to evaluate or prioritize the need for toxicological testing. There is ongoing discussion on its general applicability

for safety assessment of substances that are present at low levels in consumer products such as cosmetics or for impurities or degradation products. For the recent evaluation of the general applicability of the TTC concept by the nonfood Scientific Committees of the European Commission, see SCCS/SCHER/SCENIHR (2012b).

Regulations for Specific Chemical Classes

Jurisdictions and regulatory agencies around the world have established a variety of guidelines for risk assessment and permissible exposure standards for chemicals in the workplace, the home, and the general environment. Regulatory decision-making depends upon the estimation of health risks from chemical exposure.

Health risks of chemicals designed for specific applications, for example, consumer products, drugs, or pesticides, must be assessed when people are exposed in the many types of environment in which people can be found. Therefore all elements of risk assessment, for example, hazard identification, dose response, exposure, and the risk, have to be thoroughly evaluated.

Data requirements for **new and existing chemicals** usually depend on annual production rate and the extent of human exposure. When there is considerable exposure, regulatory requirements demand an extensive toxicological evaluation of the potential adverse effects of the specific chemical and the likelihood of their expression under the conditions of use or exposure and the definition of the MOE or the health risk under defined conditions of exposure.

For **drugs** special emphasis must be placed on efficacy, therapeutic index, potential side effects, and the effects of over-dosage.

For **pesticides** the relative impacts of the chemical on the target versus on people is a critical requirement. Thus, the NOEL for people must be established, and an ADI must be determined because of the possibility of contamination of food and other consumer products with the pesticide, and the margin of safety needs to be established.

Exposures to **chemicals at the workplace** is, accordingly to law, controlled by the Occupational Safety and Health Administration (OSHA) in the United States and by the Chemicals Law Act (1992) in Europe. Various governmental and nongovernmental institutions are involved in setting occupational exposure standards. Since the institutions publish the complete toxicologically relevant information and a justification for the proposed limit value, these documentations are valuable sources for the toxicological database of the compounds.

Table 1 provides references to institutions that publish documents on the toxicological data of chemicals.

REACH

In 1992 the European Commission estimated that about 100,000 chemicals are in use. They are produced in quantities ranging from less than one ton to several million tons produced per year. Except drugs and pesticides, data requirement for existing or new chemicals has not been regulated. Although it is the responsibility of the

producer and downstream user to release safe products, there are high volume products with a relatively small database. Several programs have been launched to obtain knowledge at least for compounds with high annual production rates. In the US EPA has initiated a HVP program. In an international cooperation the OECD has launched the ICCA program, which evaluates and documents the available information on environmental and human health hazards and risks for about 1000 chemicals. In Europe, Risk Assessment Reports under the Existing Chemical Program of about 150 compounds are being produced.

REACH (Registration, Evaluation and Authorization of Chemicals) of the European Union is to identify substances with hazardous properties and to evaluate the risks of human and environmental exposure. The regulation became effective by 2008. It is the responsibility of the producer or downstream user to provide the necessary information to the European Chemical Agency (ECHA 2017a). The extent of toxicological information largely depends on the annual production rate of a chemical. As long as there is no indication of a specific risk the chemicals will be registered for the intended use. Special attention will be paid to CMR compounds as well as to chemicals, which show bioaccumulation, persistence, and toxicity (BPT compounds) in the environment. According to the regulation the extent of information to be submitted depends on the amount produced or imported annually and requirements are highest for compounds of >1000 t/a, less for <100 t/a and lowest for 10–100 t/a chemicals.

Member states, producers, or downstream users can propose classification and labeling of chemicals and restrictions and the proposals are evaluated by the Risk Assessment Committee (RAC) of ECHA. The consequence of a CMR classification in Category 1A or 1B is a ban for consumer exposure. In such cases industry can apply for authorization for a specific use by providing evidence; there are no alternatives and the risk of consumer exposure is low. Proposals for restrictions also need to demonstrate that there are no less toxic and economically acceptable alternatives and in case the chemical is further used that the risk for consumers is not tolerable. Both the proposals for authorizations and restrictions are evaluated by the Risk Assessment Committee (RAC) and the Socio-Economic Committee (SEAC) of ECHA. The latter committee performs a cost benefit analysis for the restriction or authorization of the chemical and the alternatives.

Summary

The aim of Regulatory Toxicology is to control production, use, and deposition of dangerous materials in order to prevent adverse effects on human health and the environment. This requires sufficient information on the hazardous properties of a compound, their relevance to man and of human and environmental exposure, which is a prerequisite for appropriate risk assessment and the decision whether regulatory consequences are warranted.

There is an array of testing procedures to determine the hazardous properties such as acute, subchronic, and chronic toxicity, irritation and phototoxicity, sensitization and photosensitization, genotoxicity, carcinogenicity, or toxicity to reproduction. Information on the toxicokinetics and mechanisms of the toxic effects improve the

relevance of the findings for man. More recent methodologies like toxicogenomics, or high-throughput testing of agents for a single end-point, will become increasingly available and may improve hazard identification and aid in the identification of common mechanisms of multiple agents.

The public and the scientific community expect that Regulatory Toxicology is science based and that the proposed regulations rely on an appropriate evaluation of the intrinsic properties of an agent (hazard identification) and of the risk of a defined human and environmental exposure. Thus, the prerequisite of Regulatory Toxicology is an understanding of the principles of toxicology, the uncertainties of hazard assessment, and risk assessment and by that identification of adverse and no adverse effect levels and exposure assessment. The precautionary principle should be applied only in case of insufficient information for a final regulatory decision, not to justify elimination of exposure to any non-wanted chemicals. The TTC concept is a nominal oral dose which poses no or negligible risk to human health after a daily lifetime exposure. At such low exposures no hazard identification is considered to be necessary.

In general any regulatory decisions need to be based on an appropriate risk assessment of the possible human or environmental exposure. This can be retrospective in case of existing chemicals or prospective for newly developed compounds. According to REACH restrictions and authorizations also require a socioeconomic evaluation before the final decision.

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Working Areas of Regulatory Toxicology

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Michael Schwenk and H. Paul A. Illing

Contents

Abstract

As in other technical fields, there is increasing diversification in the toxicological risk assessments undertaken by, or on behalf of, regulatory agencies. This is reflected in the many ways in which regulatory toxicology (health and environmental risk assessment) work areas can be divided. These include by end use, by institution,

M. Schwenk (✉)
Tübingen, Germany

Formerly: Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

H. P. A. Illing
Formerly: Centre for Occupational and Environmental Health, University of Manchester, Manchester, UK

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by chemical properties, and by working methods. Although coordination is essential, different institutions sometimes make regulatory decisions independently of one another. Consistency of decision involves harmonizing; thus, cross-border cooperation of toxicologists and other regulatory affairs specialists is essential.

Keywords

Discipline · Agency · Authorities · Industry · University · Toxicology

Introduction

Regulatory toxicologists do not operate in a vacuum. There is an objective, and there are societal, legal, and philosophical contexts that underlie the scientific decision-making processes of regulatory toxicology. Setting these contexts involves other professionals and nonprofessional groups, such as citizen action committees, lobbying groups, trade associations, and legislators (politicians and lawyers). Understanding and explaining these contexts and how they operate is the role of psychologists and sociologists. Further information on this aspect of regulatory toxicology is beyond the scope of this chapter but can be found in, for example, Illing and Marrs (2009) and Illing (2009).

The expertise for undertaking regulatory risk assessments comes from toxicologists, epidemiologists, exposure specialists, and, in some cases, economists concerned with risk–benefit assessments. These may be found working in government authorities, industry, contract research organizations, and academia (Fig. 1). Each of these institutions has extended international communications networks (both to regional, e.g., European, and international [UN and OECD] bodies). Despite some competition, there is also a constructive cooperation between the institutions.

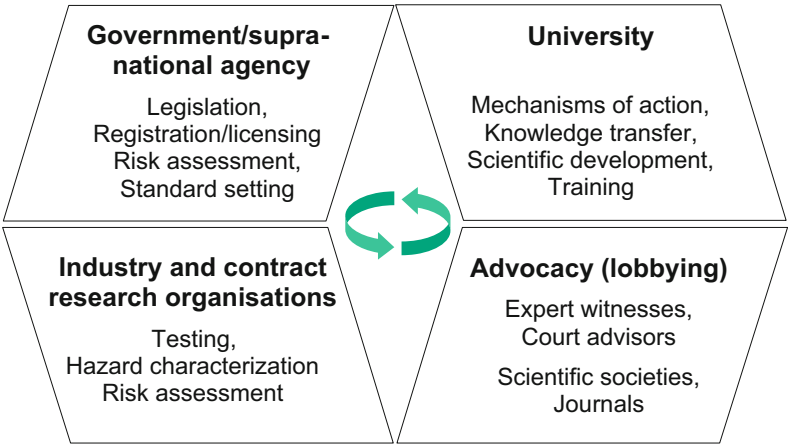

Fig. 1 Institutions

Authorities

Toxicologists (including clinical toxicologists) and other related specialists such as epidemiologists, occupational hygienists, exposure specialists, and policy makers in government (and supranational) agencies advise the authorities on various levels such as local administrations, ministries, and the government. Toxicologists are involved in the generation and monitoring of test method standards, audit procedures, and standards, registrations, and licensing procedures. Since they have to consider long-term unwanted aspects on the population and environment, they largely work on the basis of conservative risk assessments and, when dealing with environmental issues, the "precautionary principle." They use their toxicological and ecotoxicological expertise to estimate specific risks (in a risk assessment), and when the risk is not sufficiently low to constitute an acceptable risk, they may then join with others in undertaking a risk–benefit analysis in order to determine a "tolerable risk" based on trading the usefulness of a substance with the necessity of protection.

While it may be developed by individual scientists and regulatory specialists, acceptance of the relevant conceptual underpinning for this work is usually very slow and obtained via authoritative national and international bodies. Test methods and audit systems ("Good Laboratory/Clinical/Manufacturing Practice guidelines") are also developed through authoritative international bodies. Of particular importance are the OECD (Organisation for Economic Co-operation and Development), the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use), the EU Scientific Committees, and academic bodies such as the US National Academy of Sciences, the UK Royal Society, and the DFG (Deutsche Forschungsgemeinschaft). The regulation of different sectors may be (a) by sector defined by end use, e.g., pharmaceuticals, veterinary medicines, medical devices, food (including additives and contaminants), animal feed, plant protection products, and biocides, and (b) by environmental compartment – water quality; indoor, outdoor, or workplace air quality; and soil contamination. These sectors can involve different agencies, and the agencies may be largely independent of each other though they are cooperating at UN and international levels (Fig. 2). However, there are overlaps, for example, between automobile exhausts and urban air pollution. In general, good networking is required to allow for better harmonization.

Industry

Toxicologists and regulatory affairs specialists in industry have the responsibility to ensure that products placed on the market have a satisfactory risk/benefit ratio. This is of particular interest for quality conscious companies. Toxicologists in industry may commission contract research organizations (CROs) to undertake standard tests to protocols described by the authorities, or they may undertake testing "in house." Studies for regulatory purposes rely largely on internationally

Authority	*Toxicological Responsibility*
US EPA / EU National Authorities WHO	Toxicology of drinking water
EMEA (EU) / FDA (US), ICH	Pharmaceutical toxicology
Individual national or sub-national investigators (e.g. Police), UNODC	Forensic toxicology
US Defense and Homeland Security / EU National Defence and Interior Departments, OPCW	Toxicology of agents associated with warfare/terrorism
US OHSA / EU National bodies, ILO	Workplace toxicology
US FDA and Dept Agric / EU EFSA, FAO	Food toxicology

Fig. 2 Examples of toxicology-associated agencies and their fields in the USA, EU, and under international umbrella (blue color)

standardized protocols for determining the toxic potential of individual substances. These studies usually seek to identify pathological and clinical–chemical endpoints and a dose–response in animals. Investigative studies using structure–activity relationships and/or in vitro methods are increasingly important when seeking to better understand the potential toxicity. These results form the basis for the initial hazard assessment for a newly developed chemical. Exposure assessment is also conducted to see if there is a sufficient margin of exposure for the intended use. If specific risks have to be further clarified, additional experimental work related to, for example, toxicokinetics and mechanisms of action may be performed. Such nonstandard tests often require very specific methodologies and may be performed in cooperation with partners from universities or from contract research institutions. Where possible, the standardized regulatory testing is subjected to an audit process, Good Laboratory Practice, supervised by the relevant national authorities. The tests are conducted to standardized protocols, and the results evaluated using standard procedures. When dealing with products intended for human use, studies may also be conducted in volunteers, in which case they are subject to clearance by appropriate ethics committees and Good Clinical Practice. This is the main information source for the authorities, who make a regulatory decision about the registration and categorization of the compound.

Once a substance has been placed on the market, either for a specific use or more generally, there is a need for monitoring for unidentified toxic effects ("unknown unknowns"). For drugs this is called "pharmacovigilance." Through this process, it is possible to check if the risk management procedures (either for the specific chemical or use or more generally) are adequate or, if not, to reassess and reevaluate the risks.

Universities and Other Basic Research Institutions

Toxicologists at university and in basic research mainly aim at understanding toxicological mechanisms at the cellular level. They often use investigation techniques which are not subject to standardization but provide new methodological approaches and scientific knowledge. In this context, they develop novel methods that better predict toxic effects. Epidemiologists and experts in exposure modeling and measurement also contribute to the sciences underpinning risk analysis. All of these specialists must encourage cooperation with neighboring scientific disciplines and networking with regional and national partners. They often act as experts in regulatory committees. Finally, they play a central role in the education of young academics.

When there is a need for risk–benefit analysis, there is a need to environmental economists. Integrating their role with that of the other participants in the risk (or risk–benefit) evaluation is still at an early stage, and there is therefore much scope for academic research in this field.

Of increasing importance is the need for an understanding of the psychological and sociological aspects of the process of risk analysis (risk assessment and risk management) and of how the public perceives risks. It is essential that the public (as a whole) has confidence in the regulators, and a key need is an understanding of how public and regulatory understanding can be merged. Psychologists and sociologists working on aspects of risk perception offer insights into this process, and their contribution should not be disregarded.

Contract Research Organizations

CROs are often specialists in specific tests or evaluations, in which they are highly experienced. In these niches, they are likely to be more efficient and more economical than other institutions.

Advocacy (Lobbying)

Advocacy groups (such as Greenpeace, Friends of the Earth, anti-vivisectionists, trade associations) are essentially aimed at trying to persuade regulators, either directly or through persuading public opinion, that their views concerning issues should be preferred in place of those accepted by or about to be accepted by the regulator.

Expert Witness/Court and Public Inquiry Advisor Work

Generally, this work is carried out by the individual rather than by a type of institution. The focus of this type of specialist is in defined problem fields, such as

advising in litigation or in criminal prosecutions concerning causes of damage or in public inquiries into incidents/accidents. The expert witness prepares expert statements containing toxicity profiles set against information on specific incidents (and the requirements of the legislation) in order to indicate to the parties and, if it comes to Court, the Court the relevant facts and their implications. The public inquiry expert advisor advises the presiding officer (usually a judge) on the scientific facts and their implications for the inquiry.

Scientific Societies and Journals

The toxicological scientific societies are self-administered organizations of toxicologists from the different working areas. They have the main aim to promote the toxicological sciences. Scientific questions concerning how toxic agents work are traditionally the main focus of these societies.

Risk is a statistical concept that relies on toxicological data to define the hazard on the one hand and statistics (probability) to define the likelihood of the event occurring or of the exposure resulting in harm. Traditionally, scientists in universities and university-associated research units are research-oriented and not much interested in the principles and issues associated with the risk evaluation part of the regulatory process. These issues involve nonscientific aspects of risk (such as attitudes to risk and risk perception), and nonscientific aspects may prevail.

As a political process is involved, there is room for contributions from the social sciences (sociological and psychological aspects of risk, notably the influence of risk perception on risk evaluation). The ability to obtain a compromise may have a greater role in toxic risk regulation than scientific exactness. Hence, the ability to influence regulatory decisions is becoming increasingly important as an activity in which chemical and toxicological societies participate. It also provides a platform for the participation of science in international regulatory spheres and sometimes opens the door to highly interesting new ideas for research.

So it is not surprising that many scientific societies are increasingly engaging in issues of regulatory toxicology at the national and international level. They provide a forum in which basic scientists, risk analysts, and toxicologists can freely exchange ideas, without the restrictions, which they might have within their institution.

As a consequence of the recognition of this wider role for experts in regulatory toxicology, risk assessment and risk evaluation are increasingly important parts of the training of toxicologists. This is being encouraged by the scientific societies. In parallel, articles on topics involving regulatory toxicology are increasingly found in the scientific journals. This trend has been early recognized and promoted by the "International Society of Regulatory Toxicology and Pharmacology" and its journal and the foundation and development of journals in the field of risk analysis that accept articles on toxicological aspects of risk analysis.

Chemical Properties

The chemist is usually most interested in the chemical properties of a substance and will therefore find it logical to classify toxic substances according to their chemical properties. Thus, one can distinguish between the regulation of inorganic chemicals (e.g., metal toxicology), organic chemicals (many industrial chemicals), natural products (e.g., toxins), and genetically engineered products (these are a subgroup of organic molecules, usually of high complexity). A more far-reaching differentiation can be based on functional groups (nitrosamine regulation) or a chemical moiety ("dioxin" regulation). Finally, it may be crucial for the toxicological assessment whether one deals with a pure substance or a mixture (combination effects such as inhibition or synergism) and whether these are dissolved or in particulate form (e.g., dust, fine dust, nanoparticles).

The effect researcher, who may typically be a biologist or physician, is more interested in biological and medical effects. He/she accordingly arranges groups of substances with the same effect, such as allergens, irritants, initiators, promoters, endocrine disruptors, cytochrome P450 inducers, and neurotoxic or hepatotoxic substances.

The attention of toxicologists in the event of toxicological emergencies is focused on the harmful effects and the causing substances (e.g., dioxins after the accident at Seveso, fatalities after intake of novel psychoactive substances).

The legislation concerning prevention and control of accidents and emergencies may be included in the regulations concerning specified areas of use or, especially in the case of industrial processes, may be based on the scale of the potential consequence.

Regulated Areas and Legislature

It is not unusual that different levels of protection are defined for different purposes. The two principal criteria are the "broadly acceptable" criterion and the "intolerable" criterion. There may be a range of circumstances between these two criteria where a risk–benefit analysis indicates that a risk is "tolerable." Thus, for a pharmaceutical with a high positive effect (e.g., a "lifesaving" drug), it may be acceptable to take into account a certain level of unwanted effects that would be unacceptable for a treatment for a minor effect such as headache. This means that a risk–benefit analysis is applied. In the case of regulation of persistent environmental pollutants (e.g., dioxins) in the human body, one has to accept that it will take years before reduction measures, such as minimization of exposure, achieve visible success. These are circumstances where it might be appropriate to apply the "precautionary principle" and minimize exposure.

Regulations According to Application Area

When manufacturing and marketing a chemical, there is a clearly identifiable manufacturer/supplier. Both incidental worker exposure and release to the environment during manufacture can be regulated, as can the approach to dealing with major accidental releases. Product safety regulations are made according to the use to which the substance is put, with a reserve scheme for those chemicals and uses not subject to more specific legislation. Regulated uses include pharmaceuticals, cosmetics, biocides, flame retardants, food additives, industrial chemicals, radiochemicals, solvents, or chemical weapons. Regulations concerned with ambient media are more difficult to enforce as there may be no clearly identifiable source and/or they have no identifiable supplier. They are regulated by medium (air, water, soil) where it occurs.

Ambient Media

Among the regulated media are water, soil, ambient air, indoor air, workplace, food, consumer products, and human body fluids. The example of "water" can demonstrate, in how many subareas regulations of chemicals are effective: drinking water, mineral water, bottled water, water for baby food, water for injection, pool water, river water, bathing water, wastewater, surface water, groundwater, etc., each having a larger number of country-specific sub-rules. A clear demarcation between regulated uses and regulated media is not always possible.

Understanding Regulations

There exist detailed technical specifications, guidelines, and limit values associated with the control of toxic chemicals (Fig. 3). The relevant laws and regulations usually describe the levels of protection required and provide guidance on the technical rules and procedures that were applied in order to generate a guideline or a limit value. Knowledge about the background of the respective regulations and about the state of discussion among experts in the relevant area is a prerequisite for appropriate work by the regulatory toxicologists. Regulations are often updated in order to take into account new developments and insights to protect the population and environment. Much of this work is becoming international in nature. For an individual toxicologist, it is no longer possible to keep an overview of the entire width of all areas either nationally or internationally. Therefore, a division of labor is essential. But it is just as important to have an exchange between the fields and to encourage harmonization, provided that it does not impose a "drag" on the implementation of new procedures.

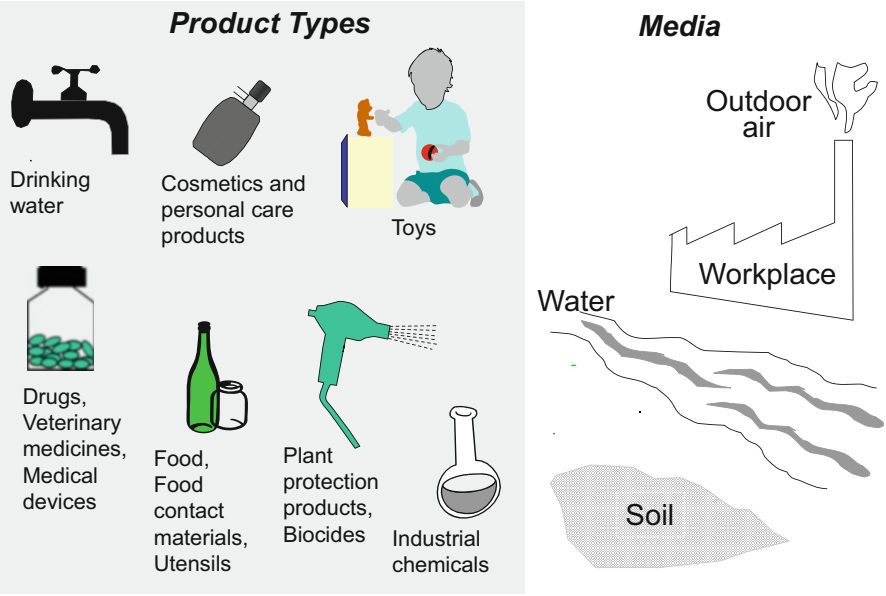

Fig. 3 Regulated products and media (examples)

Alarm Systems

There are three types of risk: "known knowns" (identifiable and quantifiable risks), "known unknowns" (identifiable but unquantifiable risks), and "unknown unknowns" (risks which have not yet been identified). Even a good regulation for the protection of workers, consumers, and the public and good management systems may not completely exclude the possibility of a toxicological accident or an unforeseen situation. This is, for example, the case, when an unforeseen rare immunological sensitivity is triggered by a compound in few individuals or when a substance is applied the wrong way. To detect such incidents, many countries have a monitoring requirement. For medicines, one such scheme is known as "pharmacovigilance," and physicians are expected to report suspicions of "side effects." The collected information is analyzed by toxicologists, who thus gain insight into the role of specific substances in incidents and can change the risk management measures (greater supervision, e.g., by restricting prescribers and outlets, improved regulation).

Working Methods

Based on toxicological data, the regulatory toxicologist considers the safety requirements for the particular use and then estimates under what conditions and to what extent the population, including predefined groups at extra risk, may be exposed to a substance,

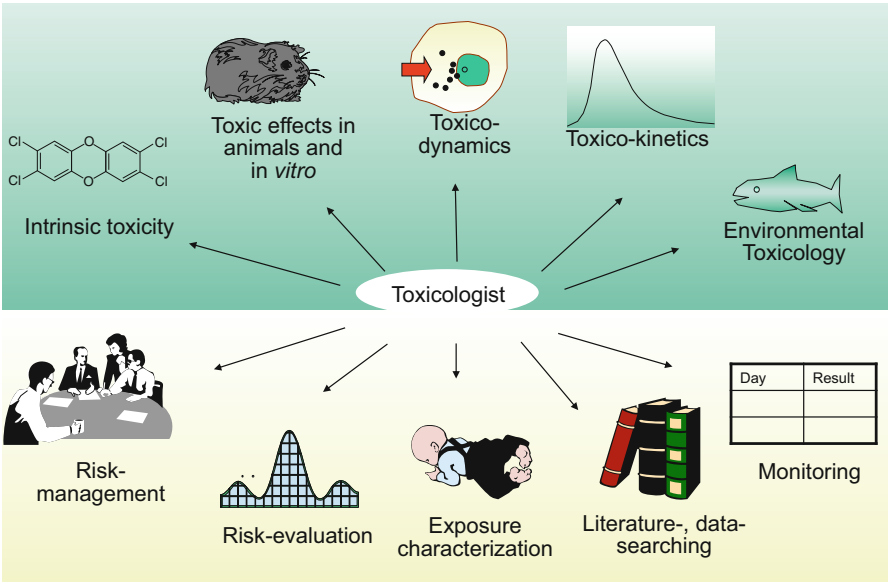

Fig. 4 Work areas in regulatory toxicology

ideally without incurring any ill health. For this task, he/she requires special knowledge and experience in the interpretation of toxicological findings, the regulatory standards, the legal framework, and the implementation process. Specifically, in-depth knowledge of the common working methods, shown in the figure, is required (Fig. 4).

In addition to that methodological experience, the regulatory toxicologist should have some technical creativity that helps to find acceptable solutions for unsolvable problems and should exhibit a high communicative competence. The latter is required, because the regulatory toxicologist must sometimes explain unpleasant findings or defend unpopular decisions in his institution or in public. In conflict situations, he must be able to defend the ethics of toxicology, explain safety standards, and discuss technical feasibility.

As in all professions, there is a hierarchy concerning the professional status of toxicologists. The experimental toxicologist can publish in esteemed journals and thus contribute to global knowledge and ensure its status among peers. The regulatory toxicologist will remain more anonymous, since his written work will normally be used by commissions, who will incorporate it in statements or in laws. This gives little scientific credit but a great deal of satisfaction due to the practical importance of his/her work.

Cross-References

► Limit Value Setting in Different Regulatory Areas of Toxicology
► Toxicological Risk Assessment in Different Jurisdictions

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National and International Collaboration in Regulatory Toxicology

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Alfonso Lampen and Klaus E. Appel

Contents

A. Lampen (✉) · K. E. Appel
Federal Institute for Risk Assessment, Berlin, Germany
e-mail: Alfonso.Lampen@bfr.bund.de; Klaus-Erich.Appel@bfr.bund.de

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Abstract

Achieving the world's social and economic objectives is not possible without the use of significant quantities of chemicals, including industrial chemicals, pesticides, and pharmaceuticals. Comprehensive and cost-effective use of these substances while maintaining high health and safety standards is, in principle, possible and has been shown in a number of cases. However, it is still a long way until these standards are implemented worldwide following the principles of sustainable development with the goal of a better quality of life of all people. A number of international bodies and authorities promote the sound management of chemicals at national and international level, some of which are described in this chapter.

Keywords

Agencies · Chemical safety · Consumer protection · ECHA · European Chemicals Agency · International collaboration · IPCS · OECD · Organizations · Stockholm Convention · United Nations · World Health Organization

Global Chemical Production

Production, trade, and consumption of chemicals are of enormous economic importance. According to the chemical industry chemicals valued at € 3347 billion were produced worldwide in 2018 (Cefic Chemdata International 2019). Since 2009, China is the world's largest chemical producer contributing 35.8% of global chemical sales in 2018. Europe (EU) is the second largest chemicals producer in the world (€ 565 billion). Although 80% of all chemicals are produced in only 16 countries, chemicals are used in all countries worldwide. Economic indicators point to a significant increase in chemical production and use in the decades to come. The BRIC countries (Brazil, Russia, India, and China) accounted for 42.8% of global chemical sales in 2018. Most of this production is still expected to take place in OECD (Organisation for Economic Co-operation and Development) countries. However, a shift to developing countries takes place in parallel.

Today, approximately 100,000 chemicals are available on the market, and many new substances are added each year. In addition, thousands of chemicals of natural origin exist. Taking into account that people can potentially come into contact with all of these chemicals, the resulting need for information concerning related health and environmental risks is enormous. Considering costs and time necessary to collect relevant toxicological information, it is only normal that the international community shares the task of data collection, especially since no country alone would probably be able, to cope with this challenge alone.

Milestones in International Chemical Safety

Chemical safety dates back to the beginning of the twentieth century. At the time, few chemicals of known risks were regulated, for example, international recommendations existed concerning the safe handling of white phosphorus in the production of matches. In fact, little to nothing was known about the risks of the majority of the chemicals produced and used at that time, and accordingly, these chemicals were not regulated. It was only decades later that industrialized countries begun evaluating and classifying some chemicals in order to inform the process of establishing safety measures. In addition, countries started to evaluate not only the risks associated with the acute health effects but also chronic, genetic, environmental, and other effects that may be caused by handling the chemicals (Somogyi et al. 1999).

International Program on Chemical Safety (IPCS)

In 1972, the United Nations Conference on the Human Environment was held in Stockholm, Sweden. At this conference, among other things, countries asked for an international chemicals safety program to serve as an early warning system to prevent disease burden associated with chemicals by undertaking risk evaluations of chemicals by applying internationally harmonized methodologies. As a result of the Stockholm Conference, the IPCS was established in 1980. IPCS is a joint venture of the UNEP, ILO, and WHO. The overall objectives of the IPCS are to establish the scientific basis for assessment of the risk to human health and the environment from exposure to chemicals, through international peer-review processes, as a prerequisite for the promotion of chemical safety, and to provide technical assistance in strengthening national capacities for the sound management of chemicals.

United Nations Conference for Environment and Development (UNCED)

In 1992, UNCED introduced sustainable development as the guiding principle of national and international environmental policy (United Nations 1992). The principles for effective international chemical safety and the sound management of

chemicals were presented in Chapter 19 of Agenda 21, the program of action for the twenty-first century, adopted by more than 170 countries. Chapter 19 contains objectives for the environmentally sound management of toxic chemicals including the prevention of illegal international traffic in toxic and dangerous products. The program areas for chemical safety in Chapter 19 of Agenda 21 include (i) international assessment of chemical risks, (ii) harmonization of classification and labeling of chemicals, (iii) information exchange on toxic chemicals and chemical risks, (iv) establishment of risk reduction programs, (v) strengthening of national capabilities and capacities for management of chemicals, and (vi) prevention of illegal international traffic in toxic and dangerous products.

International Forum on Chemical Safety (IFCS)

In 1994, the IFCS was established in response to the request by governments at UNCED. IFCS provided an open, transparent, and inclusive forum for discussing issues of common interest and also new and emerging issues in the area of sound management for governments, intergovernmental organizations, and non-governmental organizations, including from the private sector. The IFCS facilitated consensus building on issues and actions addressing chemicals safety and adopted recommendations for governments and intergovernmental organizations, including the Bahia Declaration on chemical safety in 2000. By its efforts, IFCS made an important contribution to the implementation of the Strategic Approach to International Chemicals Management (SAICM). With the adoption of SAICM, the existing of IFCS has practically ended (see below).

Interorganization Program for the Sound Management of Chemicals (IOMC)

The IOMC was established in 1995 following recommendations made by UNCED in 1992. IOMC's role is to promote coordination of policies and activities of chemical programs of international organizations, pursued jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment. FAO, ILO, UNEP, UNIDO, WHO, and OECD initially signed a Memorandum of Understanding; UNITAR joined the IOMC in 1997, the World Bank in 2010, and UNDP in 2012. The IOMC works on subjects related to those elaborated in Chapter 19 of Agenda 21 (see above) and now covered by the SAICM (Strategic Approach to International Chemicals Management) Global Plan of Action (see below).

World Summit on Sustainable Development (WSSD)

The WSSD in Johannesburg, South Africa, was held 10 years after the UNCED Conference in Rio de Janeiro and 30 years after the first United Nations Conference on the Human Environment in Stockholm Environmental Conference. Chapter 22 of

the action plan adopted in Johannesburg is addressing chemical safety renewing the commitments of Agenda 21 of UNCED for the environmentally sound use of chemicals for the purpose of sustainable development, as well as the protection of human health and the environment. WSSD targeted that by 2020, chemicals are used and produced in ways that minimize significant adverse effects on human health and the environment, taking into account the precautionary principle.

The Strategic Approach to International Chemicals Management (SAICM)

SAICM is a policy framework to promote chemical safety around the world. SAICM has as its overall objective the achievement of the sound management of chemicals throughout their life cycle so that, by 2020 meeting the WSSD goal (see above), chemicals are produced and used in ways that minimize significant adverse impacts on human health and the environment.

SAICM is distinguished by its comprehensive scope: ambitious "2020" goal for sound chemicals management, multi-stakeholder and multi-sectoral character, endorsement at the highest political levels, emphasis on chemical safety as a sustainable issue, provision for resource mobilization, and formal endorsement or recognition by the governing bodies of key intergovernmental organizations. SAICM comprises the Dubai Declaration on International Chemicals Management, expressing high-level political commitment to SAICM, and an Overarching Policy Strategy which sets out its scope, needs, objectives, financial considerations underlying principles, and approaches and implementation and review arrangements. Objectives are grouped under five themes: risk reduction, knowledge and information, governance, capacity building and technical cooperation, and illegal international traffic. The Declaration and Strategy are accompanied by a Global Plan of Action that serves as a working tool and guidance document to support implementation of SAICM and other relevant international instruments and initiatives. Activities in the plan are to be implemented, as appropriate, by stakeholders, according to their applicability.

International Agreements

Intense debates in the 1980s and 1990s led to the beginning of the adoption of a number of important conventions related to chemical safety.

Rotterdam Convention: Convention on the Prior Informed Consent Procedure for Certain Hazardous Chemicals and Pesticides in International Trade

The Rotterdam Convention prevents unwanted trade of chemicals through the application of the legally binding prior informed consent (PIC) procedure. PIC regulates the exchange of information and the shared responsibility of export and

import country in the international trade of certain hazardous chemicals in order to protect human health and the environment from potential harm. The convention deals with chemicals that are banned or severely restricted in some countries (particularly in industrialized regions) but that are still exported to other countries (particularly in developing regions).

Stockholm Convention: Convention on Persistent Organic Pollutants (POPs)

The Stockholm Convention is a global treaty to protect human health and the environment from highly dangerous, long-lasting chemicals by restricting and ultimately eliminating their production, use, trade, release, and storage. Twenty-one compounds are covered by the convention, including pesticides, industrial chemicals, and unwanted combustion by-products. Once released into the environment, POPs remain intact for exceptionally long periods of time (many years), become widely distributed throughout the environment as a result of natural processes involving soil, water, and, most notably, air. POPs are found at higher levels in the food chain, they accumulate in the fatty tissue of living organisms including humans, and are toxic to both humans and wildlife.

Basel Convention: Convention on the Control of Transboundary Movements of Hazardous Wastes and Their Disposal

The Basel Convention was adopted in 1989 to protect human health and the environment against the adverse effects resulting from the generation, management, transboundary movements, and disposal of hazardous and other wastes. It was created as a result of tighter environmental regulations in industrial countries in the 1980s which had led to the trade of toxic waste from developed to developing countries where the wastes were dumped indiscriminately, spilled accidentally, or managed improperly, causing severe health and environmental problems.

The convention regulates the transboundary movements of hazardous and other wastes and obliges its countries to ensure that these wastes are managed and disposed of in an environmentally sound manner. Countries are also required to minimize transboundary movements to the extent consistent with the environmentally sound and efficient management of such wastes and treating and disposing of wastes as close as possible to their place of generation. The export of hazardous waste to non-contracting countries is prohibited and permitted only in exceptional circumstances. The export to the Antarctic is generally prohibited.

Minamata Convention on Mercury

The Minamata Convention on Mercury was named after a city in Japan where serious health damage occurred as a result of mercury pollution. Adopted in January

2013, the convention provides controls and reductions across a range of products, processes, and industries where mercury is used, released, or emitted. These range from medical equipment such as thermometers and energy-saving light bulbs to mercury-emitting activities such as mining, cement, and energy production. Governments have agreed on a range of mercury-containing products to be banned by 2020. In addition, governments agreed to draw up strategies to reduce the amount of mercury used by small-scale miners. Furthermore, the treaty aims at controlling mercury emissions and releases from, for example, coal-fired power stations, industrial boilers, smelters, waste incineration, and cement clinker facilities.

ILO Convention Concerning Safety in the Use of Chemicals at Work

The chemicals convention of the International Labor Organization (ILO) from 1990 and its accompanying recommendation aim to improve safety and health in the use of chemicals at work, which includes the production, the handling, the storage, and the transport of chemicals as well as the disposal and treatment of waste chemicals, the release of chemicals resulting from work activities, and the maintenance, repair, and cleaning of equipment and containers of chemicals. In addition, it allocates specific responsibilities to suppliers and exporting states.

Chemicals Weapons Convention (CWC): Convention on the Prohibition of the Development, Production, Stockpiling, and Use of Chemical Weapons and on Their Destruction

This agreement of 1993 aims to eliminate an entire category of weapons of mass destruction by prohibiting the development, production, acquisition, stockpiling, retention, transfer, or use of chemical weapons by countries. Countries have agreed to chemically disarm by destroying any stockpiles of chemical weapons they may hold and any facilities which produced them, as well as any chemical weapons they abandoned on the territory of other countries in the past. A unique feature of the CWC is its incorporation of the "challenge inspection," whereby any state party in doubt about another state party's compliance can request to send an inspection team.

Vienna Convention for the Protection of the Ozone Layer and Montreal Protocol on Substances That Deplete the Ozone Layer

The Vienna Convention (1985) and the Montreal Protocol (1987) aim to protect environment and thus human health against detrimental effects of human activity which change or could change the ozone layer. Concrete objectives set out in the Vienna Convention are specified in the Montreal Protocol which is to eliminate the production and use of almost 100 substances that damage the ozone layer, including freons and halons and the production and use of methyl bromide. Substances that are

very stable in the atmosphere allowing them to reach and destroy the ozone layer which protects the Earth from the UV radiation.

International Organizations

World Health Organization (WHO)

Through the International Program on Chemical Safety (IPCS), WHO works to establish the scientific basis for the sound management of chemicals and to strengthen national capabilities and capacities for chemical safety. Main activity areas include the evaluation of priority chemicals and risk assessment, the prevention and treatment of poisonings, and the health aspects of chemical incidents and emergencies. Jointly with FAO, WHO provides the Secretariat of the Joint FAO/WHO expert meeting on pesticide residues (JMPR) and the Joint FAO/WHO Expert Committee for food additives, veterinary drugs, and contaminants. The International Agency for Research on Cancer (IARC) of WHO evaluates the cancer risks of chemicals which are published in the IARC Monographs.

United Nations Environment Program (UNEP)

UNEP provides leadership and encourages partnership in caring for the environment by inspiring, informing, and enabling nations and peoples to improve their quality of life without compromising that of future generations. The UNEP Chemicals program is the focal point of UNEP activities on chemicals and provides assistance to countries in risk assessment and reduction of hazardous substances. In addition, UNEP is providing the secretariats for SAICM, the Basel and Stockholm Conventions, as well as the Rotterdam Convention jointly with FAO (see above).

Food and Agriculture Organization of the United Nations (FAO)

FAO was founded to improve the food situation and to increase agricultural productivity. Chemical safety at FAO is focusing on plant protection products and chemicals in food. FAO has developed an international code of conduct on the distribution and use of pesticides. Other activities are dealing with the disposal of obsolete and unwanted pesticides, particularly in Africa. Together with the UNEP, FAO provides the Secretariat for the Rotterdam Convention and, together with the WHO, the Secretariats for JMPR and JECFA (WHO see also).

International Labor Organization (ILO)

Chemical safety forms part of ILO's mandate to improve occupational safety and health. In addition the Chemicals Convention mentioned above, ILO Conventions

and recommendations in the field of chemical safety are dealing, for example, with the prevention of major industrial accidents, asbestos, the working environment (air pollution, noise, and vibration), and occupational cancer. ILO's main areas of activity are the development and implementation of their conventions and recommendations, including the development, promotion, and distribution of guidelines and technical standards.

United Nations Industrial Development Organization (UNIDO)

UNIDO promotes and accelerates sustainable industrial development in developing countries and in countries with economies in transition. UNIDO promotes the development of Clean Production Centers and develops and promotes risk minimization strategies for the production of agricultural chemicals as well as the transfer of safe and environmentally friendly technologies and industrial processes.

Organization for Economic Cooperation and Development (OECD)

OECD is a non-UN international governmental organization to promote policies to improve the economic and social well-being of people around the world. The OECD provides a forum in which governments can work together to share experiences and seek solutions to common issues. Chemical safety activities aim to identify, prevent, and mitigate chemical-related environmental and health risks, to prevent unnecessary trade barriers, to optimize national resources for chemical safety, as well as to integrate economics and chemical safety policy. OECD programs are dealing with chemicals safety issues, including the testing and test guidelines and assessment of chemicals, good laboratory practice and compliance monitoring, pesticides, pollutant release and transfer register (PRTR), risk management and chemical accidents, emission scenarios, and harmonization of regulatory oversight in biotechnology. If national approaches to chemical regulation are harmonized, industry is not faced with a plethora of conflicting or duplicative requirements, governments are provided with a common basis for working with each other, and non-tariff barriers to trade are reduced. The principal tools for harmonization are a set of OECD Council Decisions which make up the OECD Mutual Acceptance of Data (MAD) system, including its OECD Guidelines for the Testing of Chemicals and OECD Principles of Good Laboratory Practice (GLP; OECD 2020).

World Bank

In its environmental strategy, the World Bank lays out its ambition to support "green, clean, resilient" paths for developing countries, as they pursue poverty reduction and development in an increasingly fragile environment. The environment strategy recognizes that while there has been notable progress in reducing global poverty, there has been significantly less progress in managing the environment sustainably,

and while developing countries will still need rapid growth to reduce poverty over the next decade, the global environment has reached a critical state that could undermine livelihoods, productivity, and global stability. World Bank's objectives in the environment strategy are to improve the quality of life in countries and to protect people's health from environmental risks and pollution to reduce the disease burden. Among others, particular emphasis is given in the strategy to reduction of exposure to toxic substances.

United Nations Development Program (UNDP)

UNDP promotes the sound management of chemicals and waste as an important aspect of their work to reduce global poverty and achieve the Millennium Development Goals (MDGs). UNDP addresses unsustainable management approaches, as well as unsustainable consumption and production patterns, including poor design and material choices. These issues are considered to be the root causes for resource depletion, waste generation and pollution, impeding sustainable human development. UNDP advocates for the integration of sound chemicals management priorities into national environmental and poverty reduction planning frameworks and helps countries access resources to improve their chemical and waste regimes.

Chemical Safety in the European Union (EU)

Previously, decision-making concerning marketing of chemicals took place at the national level. Today, nearly all these decisions take place at the level of the EU. Therefore, legislation of chemicals is largely harmonized in the EU, for example, through the requirement that National legislation must be in accordance with EU law resulting in a uniform level of protection in all Member States. Concerning toxicological testing of substances, there are, in addition to the EU harmonized methods, supranational test strategies such as the test guidelines provided by OECD (Munn and Hansen 2002).

A number of EU regulatory institutions have been established.

European Chemicals Agency (ECHA)

Established 1 June 2007 in Helsinki, Finland, ECHA regulates the technical, scientific, and administrative aspects for the registration, evaluation, authorization, and restriction of chemical substances in the EU following uniform procedures.

ECHA is central to the European REACH Regulation (Registration, Evaluation, and Authorization of Chemicals) by being the recipient and the agency that verifies the registration documents submitted by manufacturers and importers.

In collaboration with agencies in Member States, ECHA develops statements concerning the risks associated with the substances themselves as well as concerning the socioeconomic consequences associated with related risk mitigation measures (prohibition, restrictions, approvals). A network of agencies in EU Member States has been established for the implementation and monitoring of chemical safety activities within the EU. It maintains a central database and develops guidance material to assist businesses. With the acceptance of all EU Member States, ECHA defines the toxicological and ecotoxicological investigations to be carried out to describe possible dangerous properties. An appeal may be brought to Board of Appeal against decisions of the agency.

European Chemicals Bureau (ECB)

The ECB in Ispra (Italy) is the central reference for toxicological information on new and old substances within the EU. It provides scientific and technical support for the development, implementation, and monitoring concerning EU regulation, especially related to toxic chemicals. It fulfills the legal requirement to classify and label chemicals based on their hazardous and toxicological properties. It assesses the risks of industrial chemicals. Furthermore, the ECB contributes to the development and harmonization of test methods within the EU. It notifies about new substances, authorizes and evaluates biocides, and facilitates information exchange for the import and export of hazardous substances. The ECB's main partners are the corresponding scientific institutions in Member States and Norway.

European Food Safety Agency (EFSA)

EFSA's role is to provide independent scientific risk assessment advice directly or indirectly of concern to food safety and consumer protection. All stages of food production and supply are covered, and scientific assessments can be made for each stage, starting from animal feed safety, primary production, to distribution to consumers (from stable to table). Related animal and plant health issues are covered as well. EFSA's main costumer is the EC Commission which in turn can also address scientific requests of the European Parliament and Member States directly and initiate risk assessments on its own. EFSA's scientific advice is provided through scientists in a number of scientific panels. A Scientific Committee coordinates the work ensuring coherence of the scientific advice produced by the various panels.

The scientific panels are composed of independent experts of different subject areas (Table 1).

The Advisory Forum is at the heart of EFSA's collaborative approach to working with EU Members States. Its members are representatives of national food safety authorities and use the forum to advise EFSA on scientific matters, its work program, and priorities and to address emerging risk issues as early as possible.

Table 1 EFSA's scientific panels

Scientific Committee (SC)
Additives and products or substances used in animal feed (FEEDAP)
Animal health and welfare (AHAW)
Biological hazards (BIOHAZ)
Contaminants in the food chain (CONTAM)
Nutrition, novel food and food, and allergies (NDA)
Food additives and flavorings (FAF)
Food contact materials, enzymes, and processing aids (CEF)
Genetically modified organisms (GMO)
Plant health (PLH)
Plant protection products and their residues (PPR)

National Chemical Safety

The space of this chapter does not allow to describe the situation in different nations. Therefore, the example of just one country, in this case Germany, will be described.

German Federal Institute for Risk Assessment (BfR)

BfR is the German scientific authority responsible for the development of risk assessment reports and expert opinions concerning food safety and consumer protection. The development of risk assessment reports and expert opinions is based on international scientific criteria, for example, the process is transparent open and comprehensible for stakeholders, including the public, the scientific community, as well as other interested groups. In general, the results are published by maintaining the confidentiality of private data. In addition to and based on the evaluation of risks, BfR develops recommendations concerning actions to be taken to manage/mitigate the risks aimed at improving food safety and consumer protection.

In particular, the role of the BfR is to evaluate possible health risks of food and feed as well as of dietetic products and novel foods with regard, for example, to natural ingredients, food additives, and contaminants. Another role of the BfR is to assess the risks of industrial chemicals, plant protection and biocidal products with regard to human safety. It is then, for example, the responsibility of the BVL to authorize/register plant protection products, while it is the BAuA that is in charge of registration/authorization of biocidal products. Furthermore, BfR provides scientific advice to the federal ministries and the BVL, and it cooperates with a number of scientific institutions and organizations in other nations with EFSA being one main partner. Finally, BfR initiates and conducts scientific research in relation to the assessments it undertakes regarding food safety and consumer protection.

Federal Office for Consumer Protection and Food Safety (BVL)

BVL has a number of responsibilities concerning risk management. It acts, for example, as the national focal point for the European rapid alert system in the food and feed sectors (RASFF) as well as in certain sectors of product safety. RASFF warns about dangerous food and feed. In addition, BVL is responsible for the management of crises in the food and feed sectors. One aim is to make a proper risk communication and management of risks before they turn into a crisis. Among other aspects BVL's role is to warn other relevant competent authorities in Germany as early as possible about products that might cause a risk to the consumers. Furthermore, BVL provides support to the federal states to oversee the market concerning food, tobacco products, cosmetics, and any commodities as well as feed, for example, be ensuring that food surveillance is been undertaken in a harmonized way in all federal states. For that purpose, BVL hosts the European and national reference laboratory for measuring residues in food and acts as the national contact point for monitoring and measurements. Data obtained from food monitoring campaigns are sent by the federal states to the BVL for analyses, documentation, and reporting.

In addition, BVL is the responsible agency for the registration/authorization of plant protection products. The decisions of the BVL with regard to plant protection products are based on the scientific input provided by other competent authorities, for example, BfR, UBA, and JKI. Finally, BVL is the responsible agency for the registration/authorization of veterinary drugs.

Federal Institute for Occupational Safety and Health (BAuA)

BAuA is a governmental research institution within the Federal Ministry of Labor and Social Affairs. Its role is to monitor and analyze the situation with regard to occupational safety and health. BAuA advices the Federal Ministry on all matters related to occupational safety, occupational health, and workplace design. The agency provides recommendations on medical care, surveillance, and the control of occupational diseases related to working conditions. In addition, BAuA assesses possible health risks due to the exposure of chemicals at the workplace, establishes occupational exposure limits, and develops protection strategies for handling hazardous substances. BAuA is the federal authority for chemicals based on the chemical law, and it manages the national office for the implementation of the REACH regulations. It coordinates the national tasks concerning these regulations and cooperates with ECHA. According to REACH, producers and importers are required to notify new chemicals before they can enter the market. Data and information need to be submitted to BAuA on the physical-chemical, toxicological, and ecotoxicological properties, classification, and labeling as well as on the safe handling of these substances. BAuA reviews the data in collaboration with other national agencies such as BfR and UBA and shares it with ECHA at the EU level.

Furthermore, BAuA is the responsible agency for the authorization/registration of biocidal products.

German Federal Environment Agency (UBA)

UBA advises the German Federal Ministry for Environment, Nature Conservation, and Nuclear Safety on environment and health issues, particularly in the field of air pollution, noise pollution, waste and water management, soil conservation, and environmental chemicals. UBA's main role is to provide the scientific and technical knowledge for the drafting of legislation aimed at protecting human health and the environment, especially concerning the control, restriction, and ban of environmentally hazardous substances and preparations as well as genetically modified organisms. In particular, UBA has a role in the ecotoxicological evaluation of different types of chemicals. In collaboration with other federal agencies (e.g., BAuA and BfR), UBA is involved in the evaluation of, for example, pesticides, biocides, and genetically modified organisms in relation to a number of various laws, including the Washing and Cleaning Agents Act, Act on water pollutants, Plant Protection Act, Biocides Act, Federal Communicable Diseases Act, and the Genetic Engineering Act. In particular, the UBA is responsible for the evaluation of chemicals hazardous to the aquatic environment and their storage and transportation (classification of these chemicals).

Regulations concerning the sound management of chemicals and genetic engineering are essentially determined by the laws of the European Union.

Julius Kühn Institute (JKI: German Federal Research Institute for Cultivated Plants

JKI activities are in the field of plant health and nature. Its role is given by the Plant Protection Act. As the Federal Research Institute for Cultivated Plants, the JKI is dealing with the efficiency, efficacy, and benefits of pesticides. In addition, JKI has a role in the diagnoses of plant diseases, including the identification of harmful organisms and pathways and routes of infection. Methods are being developed for the detection and characterization of viruses, bacteria, and other pathogens.

JKI studies the effects of pesticides on organisms, especially on organisms present/living near crops and in adjacent water bodies (ecotoxicology); it studies the impact of climate change on pests and pest management strategies; and it contributes to the further development of integrated pest management. The latter includes the development of eco-friendly methods of plant protection, the study of natural pest resistance of crops, and the promotion of production of crops with high natural pest resistance.

Additional mandatory tasks include evaluations for the registration and use of genetically modified organisms (GMO Act).

German Federal Institute for Drugs and Medical Devices (BfArM)

The BfArM is an independent federal authority in the Federal Ministry of Health with the aim of preventing health risks by continuous improvement in the safety of

medicinal products and by risk monitoring of medical devices as well as by monitoring the legal traffic in controlled substances. Main activities focus on the authorization and registration of medicinal products on the basis of the German Medicines Act. This includes the assessment of the efficacy, safety, and pharmaceutical quality of these products on the basis of pharmaceutical, pharmacological-toxicological, and clinical studies. The license of medicinal products is limited to 5 years. Renewals are granted upon application and after new evaluation. In addition, the BfArM has a role in the registration of pharmaceuticals in the European Union with EMA (European Medicines Agency) being responsible for the evaluation of medicinal products.

After marketing, the use of medicinal products might present rare adverse drug reactions which had not been observed during clinical trials. BfArM collects and assesses reports of adverse drug reactions and decides whether the information for the corresponding drugs needs to be revised. In case where the risks of medicinal products outbalance their benefits, BfArM withdraws the license of these products. In such cases, BfArM shares the information with agencies of the European Union and the World Health Organization (WHO).

On the basis of the Narcotics Act (BtMG), the Federal Opium Agency of BfArM issues licenses for the trade in narcotics. In addition, it controls the production and/or importation of narcotic and psychotropic substances.

The term "medical devices" refers to a wide range of products, including products for diagnosis, prevention, monitoring, treatment, or alleviation of disease and injury and handicaps as well as products for the replacement or modification of the anatomy such as pacemakers, X-ray, radiotherapy, and surgical instruments as well as in vitro diagnostic medical devices, prostheses, artificial teeth, etc. Health risks related to these products need to be reported to the BfArM by manufacturers, operators, and users. In turn, the role of the BfArM is to provide recommendations for risk mitigation.

Cross-References

- ▶ Institutionalized Participation in Regulatory Toxicology
- ▶ International Regulation of Toxicological Test Procedures
- ▶ Limit Value Setting in Different Regulatory Areas of Toxicology
- ▶ Toxicological Risk Assessment in Different Jurisdictions
- ▶ Working Areas of Regulatory Toxicology

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The Regulatory Process in Toxicology

4

Dietrich Henschler and Wolfgang Dekant

Contents

Abstract

The regulation to avoid or reduce potential health and *environmental risks* due to chemicals or physical factors in Germany, the European Union, and worldwide carries extremely heterogeneous features. Fundamental differences are encountered not only with regard to institutional responsibilities but also – and in particular – to nomenclature(s); definition of aims of protection; types of organization; scientific basis and extent of justification, implementation, and controls; as well as the legal status. The situation is even more complicated by interfering mandates. The system suffers from a crisis of credibility. However, recent efforts toward harmonization gain pace.

D. Henschler · W. Dekant (✉)
Formerly: Institute for Toxicology, University of Würzburg, Würzburg, Germany
e-mail: dekant@toxi.uni-wuerzburg.de

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Banning · Commission of experts · Nomenclatures for limit values · Participation · Proctecive procedures · Restriction · Scientific evaluation · Threshold Limit Values · Transparency

Possibilities of Regulation

On principle, there are four organizational types of regulation: *banning of production, sale, and uses of toxic materials*; *restrictions on use*; *mandatory use of protective procedures to avoid/reduce hazardous exposure(s)*; and the introduction of *health-based environmental threshold limit values*. Banning of production is only realized for materials with very high hazard potential. As voluntary *withdrawals from the market* by producers, bans regarding production or import (e.g., 2-naphthylamine, PCBs, pentachlorophenol) may be reduced in their effectiveness by imports due to globalization and removal of trade barriers. Bans are also excluded in case of materials which cannot be waived due to technical reasons, are formed by transformation processes in the environment, or have natural sources (such as heavy metals). In these situations, more and more preference has been given to the development and introduction of *alternative compounds* which are designed to avoid undesirable properties such as high stability in the environment. While intelligently designed alternatives may have significant advantages such as reduced potential for specific toxicities, complete toxicological data and experience from practical use of such alternatives are often not available; thus, other potential risks may be present. Another domain is *restrictions in practical applications* – a field of activities more for administrators than for toxicologists. *Protective measures* in loco (exposure prevention by personal protective equipment or using closed processes) are mostly dealt with by specialists in occupational toxicology. The most important protective instrument is the establishment and application of *threshold limit values (TLVs).* They constitute the most frequently used method of health-based protection. Therefore, the following description will focus on such limit values.

Threshold Limit Values (TLV)

Threshold limit values (and environmental standards) are maximum permissible concentrations of chemicals (and physical stressors such as electromagnetic radiation) in specified environmental compartments, in specific tissues of organisms, or in excretion products. They are presented in the form of definitive figures, expressed as mass/volume, mass/mass, volume/volume, or doses in the form of mass/time. In case of *physical stressors* (radiation, noise, heat, pressure), physical quantities are valid accordingly. Such *official limit values* are established in laws, enactments, or regulations. They are either to be adhered to or function as recommendations. *Nonofficial limit values* are established by private institutions in the form of recommendations,

which may or may not be taken over in legal technical rules (e.g., *MAK values*) (DFG = German Research Association, VDI = Professional Organization of Engineers, DIN = Administration for Technical Norms).

Stock-Taking

According to a systematic analysis performed by the Expert Council for Environmental Questions in Germany, there are more than 150 types of limit values in Germany alone. Chronologically, these were first developed for pharmaceuticals. The first dose limit for a pharmaceutical was introduced by the official German Pharmacopoeia (second edition in 1882) in the form of a maximum single or daily dose. The first limit values for workplace exposures to chemicals were introduced in 1886 (K.B. Lehmann). The numbers of limit values for chemicals in occupational or environmental settings were steadily increasing since 1960 with an exponential tendency, often enforced by increasing public pressure. More recently, ca. 20% of the derived limits each account for victuals and soil, ca. 10% each for air and water, and less than 10% each for chemicals, noise, and radioactivity. Human health is the predominant aim for the protective measures and presents 93%, followed by general protection of environment (19%), plants (16%), and animals (14%) (in part repetitive counting). Regarding the legal status, 50% each are introduced as official and nonofficial standards. At least 30 different nomenclatures are in use (see Table 1).

The authorization for the organization of work to be performed to justify a derived value varies widely, from multidisciplinary recruited commissions or committees, down to the desk of a single clerk of an agency. This confusing complexity is, in its major proportion, due to the historical development: different academic disciplines picked up, mostly incidentally, a problem and made use of their

Table 1 Designations of threshold values as used in 154 German systems of regulation of hazardous materials (according to SRU = Council of Experts of Environmental questions, 1996)

Environmental values	Unhesitating values
Tolerance values	Maximum values
Maximum tolerance values	Precarium values
Scrutiny values	Background values
Encumbrance values	Input values
Hazard suspicion values	Target values
Interference values	Acceptance values
Intervention values	Adjusting values
Action values	Coordination values
Occasion values	Damaging values
Restoration values	Threshold values
Alarm values	Preliminary values
Release values	Hesitation values
Release threshold	Environmental standards
Orientation values	Toxicity values
Scruple values	

categories of reasoning and evaluation, thus paving the way for a great variety of experience and competence. Since approximately two decades, increasing criticism of status and further development is arising, mainly driven by the interest of industry and jurisdiction to achieve reliability for planning and legal status. The lack of clear-cut targeting and rules of procedure induced activities to improve harmonization, standardization, and simplification. As a result, useful and intentionally calibrated criteria have been elaborated (SRU = Council of Environmental Questions 1996); a new commission for risk evaluation has been charged with establishing and handling uniform rules.

Profiles of Demand

Regulatory processes are understood as political decisions – ideally in the form of consensus – based on scientific assessment of potential risks, under adequate participation of societal groups. The substantial elements of demand are:

1. *Participation of the public* before and in the course of procedures
2. *Complete transparency of all steps of procedure*, e.g., publish intentions and timing
3. An essential element of transparency is to be seen in the obligation of a detailed *justification* of
4. *All scientific evaluations and proposals for regulations and decisions* in the form of detailed documents which should be available to everybody
5. Concerned *societal groups should be involved* in the discussions for the preparations of decisions
6. Accomplished decisions, particularly regarding the level of a standard, need to be enforced by validated analytical methodology to warrant *compliance*

A new element has been introduced later: obligation of *continuous reevaluation in predetermined intervals*, taking into consideration new scientific data and eventually changes in sociopolitical principles.

Procedural Steps

The profiles of demand require the integrated cooperation of expertise of different scientific domains, making the process of regulation a multidisciplinary task. The evaluations to be performed require working elements of different groups of experts. This necessitates a *sequential procedure* of defined steps, which allows for recourses from one step to each other. A model of sequential steps is presented in Fig. 1.

The process starts with the determination of *objects of protection* (targets) (human beings, plants, soil, etc.) and with *aims of protection* (e.g., complete elimination or gradual reduction of risk). Right and duty of making proposals is not restricted to governmental institutions but open to everybody. The decision about the aim(s) of

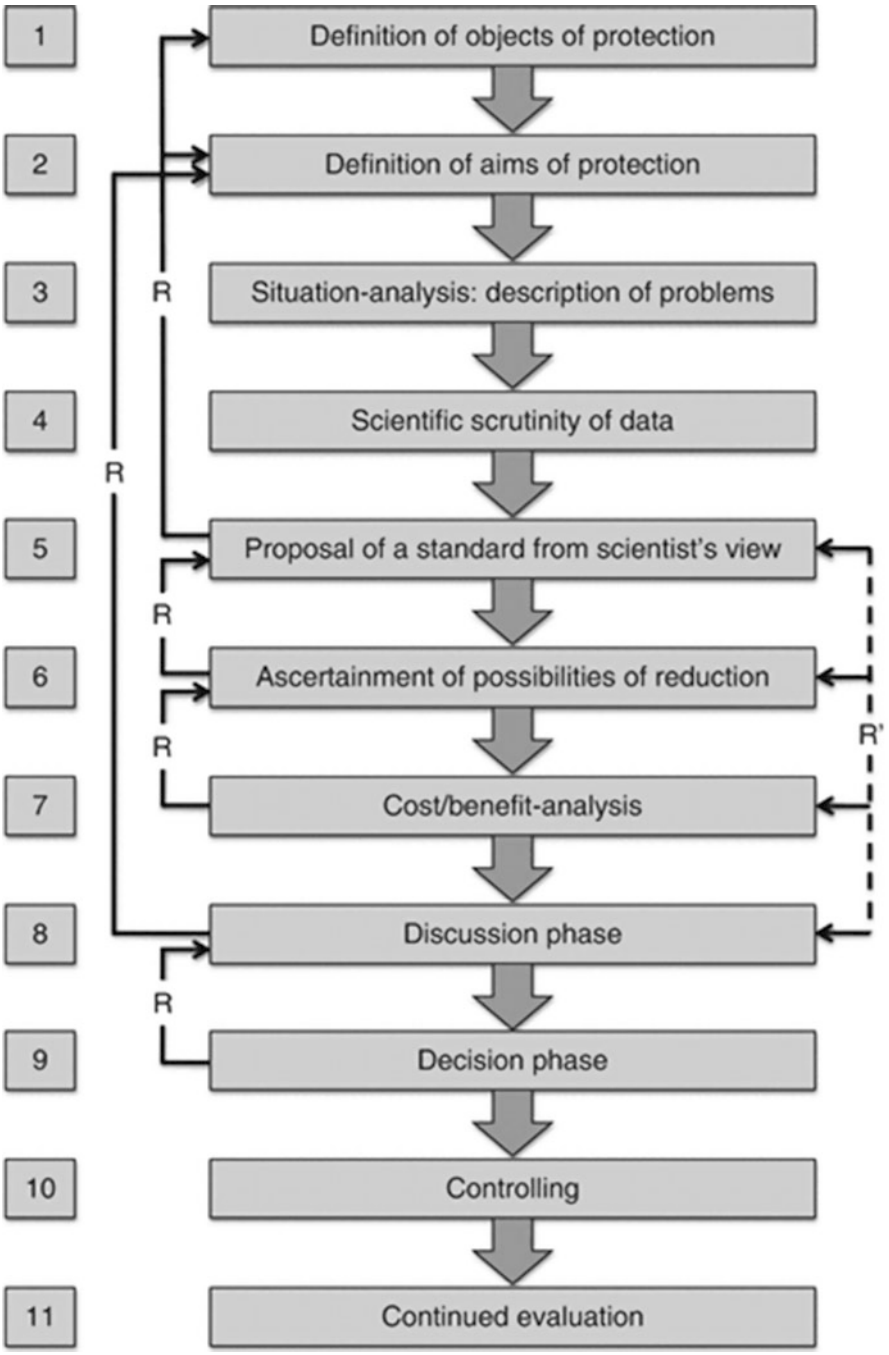

Fig. 1 Scheme of sequential progress in the form of an ideal model of steps in the regulatory process (R, R′ = checkback; SRU 1996)

protection is bound to the duty of detailed justification. This is followed by a *scientific analysis*, including a risk evaluation mostly based on published data on toxicological information or results of targeted toxicity test. Normally, a *proposal for a standard* is elaborated by the group of scientists who evaluated the data as a result of the critical evaluation of all data for which a detailed justification is mandatory, including the identification of gaps of knowledge. This step is followed by the ascertainment of *possible technical reduction of risk(s)* (often called "status of technology"), as well as the elaboration of a *benefit/risk analysis* and a *cost analysis*, both steps involving experts in engineering and economy. Again, these proposals have to be justified in detail.

After these basic steps have been accomplished, a *discussion phase* tries to set a starting point for a solution, may be in the form of several alternatives. Participants are societal groups (producers, users, employers); for checkback questions, scientists who participated in the foregoing steps should be available. The guidance of the discussions should be handled by those responsible for the (final) decision-making (governmental and/or nongovernmental). They should prepare, in the following *decision phase*, the finalized version of the standard proposal, including the detailed justification, and put through the final decision. The same group of participating experts shall also prepare the operational steps of *control of compliance* to the standard and for a *continued reevaluation* in predetermined intervals, taking into account new developments in data production and interpretation. For this purpose, a new standard necessitates the provision of suitable analytical methodology according to internationally accepted rules.

Historic Developments

The classical form of organization of the process of regulation is the *commission of experts.* This has a long tradition in Germany, particularly by the DFG (German Research Association) who, according to their statutes, provides recommendations for health-related issues. Since 1952, DFG has established so-called Senate Commissions in different domains of regulations (occupational toxicants preparing MAK values = maximum tolerable concentrations, plant-protecting chemicals, foodstuffs, cancer research, etc.). The MAK Commission has held a pilot function for many other commissions. For ambient air pollution regulations, numerous commissions have been established and are still active in the VDI (Union of German Engineers). In addition, governmental agencies – from federal down to community level – have established their own committees for giving advice in environmental problems or setting standards of their own. Some are working permanently, some ad hoc only; the latter ones suffer, in some cases, from a lack of consistency and continuity.

Membership in these commissions of scientists in general, and of toxicologists in particular, should be based on independency in their professional activities and reasoning. There is a legal basis for proving the evidence of independency in the form of official rules of administration: new members of a commission have to declare by signature that they do not hold contracts with industry, share holding

included. In this context, there remains an open issue of membership of professionals in industry: on the one hand, they may contribute a high amount of special knowledge and competence, and they may contribute to the process by submitting valuable data (sometimes unpublished) and by specific experience. One way out of this conflicting situation may be seen in having them participate by seat but not by vote. But this certainly is not satisfactory to everybody. The agencies should create clear regulations referring to this sensitive point, now and forever.

Finally, there remains one important question to be solved: Who should participate in which sector of the regulatory process and who should take which part of responsibility? Two models are in operation: (1) Unitarian, every member of the commission participates in all steps of the procedure, participates in voting, and thus carries full responsibility. (2) Separatistic, the activities in the scientific analysis, discussion, and decision are strictly separated from each other, which means everybody participates just in that sector where he/she is professionally competent and thus takes responsibility just in that part. The separation shall avoid influences upon the scientific evaluation and decision by members of interested societal groups. Further development indicates preference of the separatistic model. However, the lawmakers in Germany have not yet taken decision toward a clear and comprehensive regulation of this issue.

Types of Organization

Similar processes as those described above for Germany have been developed on the level of the European Union and internationally. However, within the different legislative contexts, the involvement of scientific expert committees varies between the sole responsibility of the advisory group regarding limit values developed to an advisory role after the value has been defined by a regulatory authority.

For example, panels of the *European Food Safety Authority* (*EFSA*) and Scientific Committees of the European Commission with specific legislative mandates develop tolerable limits for food additives, food contact materials, food contaminants, or cosmetic ingredients based on scientific principles for health risk assessment and carry the sole responsibility for the process. In contrast, in the Registration, Evaluation, Authorization of Chemicals (REACH) process, the manufacturer or importer of a chemical (registrant) is responsible for performing risk assessment and for developing tolerable exposure following specific and detailed guidance outlined in REACH regulations. The Agency (ECHA – European Chemicals Agency) interacts with the registrant and can require specific information to address issues identified in the derived exposures and potential uncertainties in the evaluation. However, due to resource constraints, it is expected that only a limited number of the submitted registration dossiers will be evaluated in great detail.

In addition, a significant role of scientists employed by regulatory agencies (governments) in risk assessment is also frequently observed. In many cases, scientific advisory boards have the role to provide comments to the developed documents regarding risk assessment. For example, in the United States, many

regulatory decision documents regarding chemical safety are drafted by regulatory agencies, and conclusions are presented to a scientific advisory board and the general public requesting comments on the conclusions.

Cross-References

- ▶ Hygienic Versus Toxicological Approaches in Regulation
- ▶ Limit Value Setting in Different Regulatory Areas of Toxicology
- ▶ National and International Collaboration in Regulatory Toxicology
- ▶ Prohibition and Restrictions in Regulatory Toxicology

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Quality Assurance in Toxicology

5

Klaus-Michael Wollin, Stephen Harston, and Werner Lilienblum

Contents

Abstract

Reliable data are essential for the assessment and evaluation of the toxicological characteristics of chemical substances and of safe exposure levels for man and the environment. Data reliability is closely linked with the exclusion or minimization of errors and mistakes in the generation of data. These objectives can be reached by the implementation of appropriate Quality Assurance (QA) systems as an

K.-M. Wollin (✉)
Formerly: Public Health Agency of Lower Saxony, Hannover, Germany

S. Harston
Formerly: Head of GLP Quality Assurance, Hoechst AG (1977–1998), Hofheim am Taunus, Germany
e-mail: stephen@harston.de

W. Lilienblum
Hemmingen, Germany

Formerly: Lower Saxony State Authority for Occupational Safety and the Environment, Hannover, Germany

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important part of the Quality Management (QM). The major characteristics and differences of the more important quality assurance systems are presented in this chapter.

Keywords

Quality Management · Quality Assurance · GxP · Accreditation · Certification · "Codes of Conduct"

Introduction

Quality must be defined in advance: The quality of a finished product or of a service function at the end of a value creation chain is determined by a number of factors – the basic elements of quality first described by Kaoru Ishikawa (1968) and linked by him into a "fishbone" or "cause-and-effect" diagram. These factors include management, environment, methods, machines, materials, measurement, and – last but not least – people. "Quality" in this context is so defined that any activity, performance, or technical product should meet specific, predefined requirements and characteristics on completion. In order to reach and maintain such quality requirements, specific prerequisites and boundary conditions on the road to the finished product must be defined in advance. These will include quality criteria and quality control procedures applicable not only to the end product but also for all critical initial parameters and intermediate steps. In the case of reproducible or frequently repeated activities, such prerequisites and boundary conditions are often defined in Standards, Guidelines, or Directives according to the specific legal and administrative systems. This applies to the majority of physical, chemical, and biological-medicinal measurement systems and to methods for the generation of data relating to chemical substances and their properties.

Quality of Data

Relevant and reliable data are required to assess and evaluate the toxicological characteristics of chemical substances or of exposure levels. The quality of the available data is of decisive importance and thus has to be carefully considered during the human health risk assessment process. Good quality means not only that the data provide an important or significant contribution in the sense of providing new insights or filling a previous gap of knowledge but also that the data is reliable, in the sense that both the probability of errors occurring and the extent of any which may occur are as small as possible.

Practically, every measurement (no matter how accurate) or other form of experimental or epidemiological data collection implies some risk of random or systematic errors, which then result in a deviation from the "true" value (which is – in general – not known). An important aim of any institution generating such data must thus be

to implement appropriate general conditions and control procedures so that there is a high probability that the data obtained approach the "true" value and can be confirmed – either by repeating the process or by some other method. Given a certain process or method, the probability of approaching the "true" value can thus only be improved by systematically eliminating all known sources of error and – gradually – identifying and eliminating unexpected or previously unimaginable sources of random and systematic error. Data quality in terms of reliability thus depends on the systematic elimination of sources of error. This necessitates a Quality Management approach with a suitable Quality Assurance system.

Quality Management (QM) and Quality Assurance (QA) (QM/QA Systems)

The aim of *Quality Management* systems is firstly to ensure that errors in ongoing processes are excluded as far as possible. As part of a continuous learning and improvement process, any remaining errors should be identified, documented, and avoided in the future. This can be achieved by the choice and implementation of a QA system with appropriate boundary conditions, methods, and controls. The international standard ISO 9001:2015 is the most prominent approach to quality management systems, specifying requirements for QM systems.

The aim of every *Quality Assurance* system is to generate credibility and confidence in the reliability of the data internally and externally – that is within the organization, toward direct clients, and all others who may be interested in the data concerned. In practice, two different strategies can be identified, neither of which alone is sufficient but which supplement each other in various QA systems with varying degrees of emphasis on individual features.

First Strategy: Traceability and Transparency of Studies

Data are usually generated in the course of experimental or in silico studies and any kind of projects. Many such studies cannot be easily repeated, should doubt about the reliability of the data arise. Reasons may be ethical grounds, cost grounds, or the huge workload involved. Examples of such studies are long-term experimental studies in animals (often with large numbers of animals), studies in human beings, and field studies with crop protection agents. Any attempt to reconstruct such studies shortly or long after they have been conducted requires extensive and detailed recording of all initial conditions, methods, working steps, and the results obtained. In such cases, an extensive documentation and archiving system is required, such as that particularly described in the *Good Laboratory Practice* (GLP) system. The workload for the testing facilities and test sites involved with such systems is significant, even for relatively small or short-term studies.

Second Strategy: Reproducibility and Comparability of Data

Ideally, experimental toxicological research produces statistically significant effects that can be interpreted as causal effects. Naturally, every single experiment is a singular observation. In order to assume a generally valid causality, individual observations should be reproducible. The requirements to obtain reproducible data in toxicological studies have been intensively discussed in recent years (e.g., Miller 2014; Briner and Kirwan 2017). Many studies to determine, e.g., physicochemical properties of substances such as melting or boiling point or the presence of substances in biological matrices can be fairly easily and quickly repeated under the same methodological conditions or can be easily checked by other means. This applies to the majority of chemical-bioanalytical and many other physicochemical determinations. The stringent application of the International System of Units (SI) facilitates comparison of data. This implicates increased requirements regarding technical expertise, calibrations, and comparison measurements (e.g., participation in inter-laboratory tests) for data validation and quality management procedures in the laboratories concerned. However, the documentation effort is then reduced and more flexible. Quality assurance systems of this type include accreditation and – for products and services – certification.

Good Laboratory Practice (GLP) and Other "GxP" Systems

Some quality assurance systems are required in relevant laws and regulations and thus fall under legal controls, for example, those for *Good Laboratory Practice* (GLP). The first GLP regulations were issued by the US Food and Drug Administration (US FDA) in the late 1970s after irregularities were discovered in the planning, conduct, and reporting of animal safety studies submitted in the registration dossiers for medicinal products (U.S. FDA 1978). Similar regulations were subsequently issued by the US Environmental Protection Agency (US EPA) covering studies conducted with pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and other chemical substances under the Toxic Substances Control Act (TSCA) (U.S. EPA 1983). The need to comply with these regulations acted as a nontariff barrier to international trade in such substances, which led the *Organisation for Economic Co-operation and Development* (OECD) to develop internationally harmonized "Testing Guidelines" and "Principles of GLP" which were then recommended for worldwide use to ensure the Mutual Acceptance of Data (MAD) generated according to the Testing Guidelines and GLP Principles (OECD 1981). The GLP Principles were recommended for use within the European Communities in 1987. The GLP Principles represent general quality requirements for the conduct of studies, and the OECD Testing Guidelines form the basis for the scientific or methodological approach. Both the GLP Principles and the OECD Testing Guidelines are the common basic concept to ensure the data quality of studies, for trust building and for the Mutual Acceptance of Data worldwide. At the end, it is up to the competent authorities to evaluate the study data with regard to

reliability, relevance, and adequacy. The latter term is used for the comparison of studies. Evaluation criteria for these three terms have been developed (Klimisch et al. 1997) and refined. For a recent overview, see, for instance, Beronius et al. (2018).

The GLP Principles (and the Testing Guidelines) are reviewed on an ad hoc basis by OECD Expert Groups and – where appropriate – revised to ensure best scientific practices. The last revision of the GLP Principles took place in 1995–1996, and the Revised Principles were formally adopted by the OECD in 1997. The Revised Principles were adopted in the European Communities in 1999 and are now binding within all Member States (in Germany, e.g., as Annex 1 to the Chemicals Law (Chemikaliengesetz)). The OECD Testing Guidelines have also been implemented into the European legislation and are being continuously updated according to the technological progress (Regulation (EC) No 440/2008). The European Regulations and Directives relating to biocides, chemical substances, cosmetics, detergents, feeding stuffs, foodstuffs, medicinal products, medical devices (where applicable), novel foods, and veterinary products all require that at least some of the test data required for the registration or regulatory approval of such products for use within the European Union be generated in compliance with the GLP Principles or with equivalent standards (EC website 2020).

The OECD has also developed procedures for governments on the inspection and verification of good laboratory practice in order to monitor the compliance of testing facilities with the GLP Principles (first adopted 1983, first revision 1989, second revision 1995). These documents have also been implemented by the individual Member States within the European Union (Directive 88/320, now replaced by Directive 2004/9 of March 2004). In addition, the OECD has sponsored the preparation and publication of a series of *Guidance Documents for Compliance Monitoring* (No. 2, 3, 9, 20), *Consensus Documents* (No. 4, 5, 6, 7, 8, 10, 13), and *Advisory Documents* (No. 11, 12, 14, 15, 16, 17, 19) providing further comments and explanations on certain specific items of the GLP Principles (for instance, quality assurance, laboratory supplies, field studies, short-term studies, computerized systems, full listing available on OECD website (OECD 2020)). These documents have no legal force but are – in practice – regarded as "state of the art" and are widely used by test facilities and test sites as well as by compliance monitoring authorities. The OECD Position Paper No. 18 *Regarding the Relationship between the OECD Principles of GLP and ISO/IEC 17025* states that laboratory accreditation (see below) is not applied to non-clinical health and environmental safety testing because ISO/IEC 17025 does not contain all of the requirements of the OEDC GLP Principles. Nevertheless, laboratory accreditation can make a valuable contribution within the GLP compliance structure. Although common rules and more detailed regulations exist, there may be differences in their interpretation, application, and enforcement between countries and even between monitoring authorities in the same country. For instance, whereas Seiler (2005) describes the implementation and application of the GLP Principles from a more "European" point of view, the same GLP Principles may be in part differently interpreted and applied in the United States and even between the two monitoring authorities US FDA and US EPA (Weinberg

2003; U.S. FDA 2020). The European Commission provides support under https://ec.europa.eu/growth/sectors/chemicals/good-laboratory-practice_en.

Good Manufacturing Practice (GMP) is a QA system (also first developed in the United States) to control in particular the manufacture of medicines, veterinary medicines, and medical devices. GMP is also prescribed by law for the manufacture of food contact materials and cosmetic products. The application and monitoring of GMP requirements is also largely harmonized, within Europe initially (1989) as "Guidelines to Good Manufacturing Practice," subsequently by Commission Directives 91/356 and 91/412 and Directive 2003/94. For details, for example, see also the websites of the European Medicines Agency (EMA 2020) and the European Food Safety Authority (EFSA 2020).

Good Clinical Practice (GCP) provides a quality assurance system for planning, conducting, and reporting clinical studies carried out – for example – to provide data in support of applications for marketing authorizations for medicinal products. The requirements were first developed by an expert working group of the "International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use" (E6(R1) Step 4 version (ICH 1996), current update: E6(R2) Addendum Step 4 version (ICH 2016))) and adopted by the regulatory bodies in the European Union, Japan, and the United States. In 2015, ICH has renamed itself as the International Council for Harmonisation and has become a legal entity under Swiss law. Among other issues, the GCPs require that clinical studies be planned and carried out according to the ethical standards described in the World Medical Association "Declaration of Helsinki." Further information on GCP can be found, for example, on the website of the European Medicines Agency (2020).

Accreditation and Certification

Most accreditation and certification systems are based on voluntary participation and are not governed by legal requirements. However, the use of such systems is often a prerequisite before a facility or laboratory may conduct studies if the results should be used in legally controlled activities. This applies, for instance, to laboratories performing analyses for the control of foodstuffs, the monitoring of ambient air or drinking water quality, or measurements to be used as part of health and safety requirements in the working environment as laid down in ISO/IEC 17025. Both systems give high priority to the use of appropriate quality management procedures. *Accreditation* is a system to monitor and approve the competencies of testing laboratories and their Quality Management systems. The organizations issuing such approvals are – themselves – monitored and accredited by the so-called Accreditation Bodies, as laid down in the International Standards Organization (ISO) Standards ISO/IEC 17011 and – when appropriate – 17020–17025. For instance, in Germany, the *Deutsche Akkreditierungsstelle* (DAkkS) is the national Accreditation Body (since January 2010). Pursuant to Regulation (EC) No. 765/2008, DAkkS acts in the public interest and as the sole provider of accreditations in Germany. *Certification* according the international standard ISO 9001:2015 relates to the

quality of products and/or service functions in the sense of a guarantee that certain defined characteristics are provided by the product or function. ISO 9001:2015 is compatible with other management systems standards and specifications, such as ISO 45001 *Occupational Health and Safety* and ISO 14001 *Environmental*. Appropriate certification and the establishment of a quality management system according to ISO Standard 13485 for medical devices is – for example – a prerequisite for the use of the CE Mark on certain types of products to be placed on the market within the European Economic Area (EEA). Another example is ISO 22716 for the manufacture of cosmetic products.

"Codes of Conduct" and Quality Assurance

A number of scientific societies and professional associations (e.g., those for medical practitioners, pharmacists, or toxicologists) have developed codes of conduct which are binding on their members. These Codices contain certain elements which help toward a quality assurance but are – usually – directed to ensuring a responsible and ethical behavior in professional activities. Such elements, for example, a requirement for scientific honesty, are important but alone cannot be regarded as a quality assurance system. The concept of "Safeguarding Good Scientific Practice" has been developed by some major institutions for basic research in response to spectacular cases of scientific misbehavior or fraud. For example, the German Research Foundation (Deutsche Forschungsgemeinschaft) (2019) has recently updated its "Safeguarding Good Scientific Practice" with some 16 detailed recommendations and suggested their use in scientific institutions, particularly those in academia. Among the more important recommendations in the sense of quality assurance are those related to organizational structure of working groups and the need for complete documentation and long-term archiving of important primary data; however, it is unclear in how far these recommendations have been or are being followed by the institutions concerned.

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► International Regulation of Toxicological Test Procedures
► National and International Collaboration in Regulatory Toxicology
► Principles of Analytical Chemistry for Toxicology
► Quality Criteria for Primary Literature in Toxicology

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Toxicological Risk Assessment

6

Maged Younes and Nikolaos Georgiadis

Contents

Abstract

Risk analysis encompasses the scientific review and evaluation of all relevant scientific data on the toxicity of, and the exposure to, a certain compound or mixture. To enable a systematic analysis of the different types of information needed, various risk analysis paradigms have been developed. Among these, the scheme developed in 1983 by the US National Academy of Sciences (NAS) has been the most widely utilized. Risk analysis provides the scientific basis for regulatory actions within the context of risk management.

M. Younes (✉)
Global Publich Health, Mies, Switzerland

Formerly: Food Safety and Zoonoses, formerly:World Health Organization (WHO), Mies, Genéve, Switzerland

N. Georgiadis
Toxicologist, European Chemicals Agency (ECHA), Helsinki, Finland
e-mail: nikolaos.georgiadis@echa.europa.eu; nikolaos.georgiadis@gmx.com

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Keywords

Adverse-Outcome-Pathways (AOP) · Hazard · Risk · Risk analysis · Risk assessment · Weigh-of-evidence (WoE)

Introduction

The term "risk analysis" is not used in a uniform manner. In some instances, the term is considered to have the same meaning as "risk assessment," while some institutions, as is the case with the Codex Alimentarius Commission, employ the term to describe the broader concept of risk regulations, encompassing risk assessment, management, and communication. For others, risk analysis is seen as the mathematical analysis and quantification of risks. Given these differences in using the term risk analysis, a clear, uniform definition cannot be given. For the purposes of this chapter, risk analysis will be described as the broader process encompassing the scientific assessment, management, and communication of risks.

Why Risk Analysis?

The toxicity of a given substance can be defined as its ability to harm living organisms. This is an inherent characteristic of any compound and will only be expressed as a function of the dose as described already by Paracelsus. Thus, any compound can be toxic if a certain threshold of exposure is surpassed. This is the reason why a distinction between "toxic" and "nontoxic" or "harmful" and "safe" substances makes no sense. In fact, the toxicity of a given substance cannot be defined without reference to the administered/absorbed amount (dose); the route through which the exposure and distribution of the substance take place (e.g., by inhalation, ingestion, dermal absorption); the level, frequency, and duration of exposure; the type and grade of the damage caused; and the lag time required to illicit the toxic effect.

It is only once the potential to cause harm and the probability of a damage are known that options to reduce/eliminate potential harm can be assessed and regulatory action be taken (risk management). Such measures need to consider other factors besides the scientific evaluation of risks, for example, socioeconomic impacts and the risk-benefit relation. The aim of risk management is to avoid risk or, if this is not possible, to reduce it as far as achievable. The basis for meaningful risk management decisions remains, however, a thorough characterization and evaluation of scientific data on toxicity and exposure: risk assessment.

Steps in Risk Regulation

In the scheme of the German Risk Commission (Deutsche Risikokommission), risk regulation encompasses the whole societal process of dealing with risks. Ideally, the process should cover three areas of risk analysis: risk assessment, risk evaluation, and risk management.

Risk Assessment

Risk assessment is the process of identifying and quantifying the potential harm due to a certain exposure to a substance (risk). Normally, it targets individuals, but there are several instances in which population risk is assessed. To accomplish this task, knowledge about toxicity and exposure, but also information on the dose-response (or exposure-effect) relation, and target populations including vulnerable groups is required (see below).

Risk Evaluation

Risk evaluation bridges risk assessment and risk management. It encompasses a value judgment of the risk posed by the substance under consideration. Questions addressed here include whether or not the risk is higher than seen with other comparable compounds, what the risk-benefit ratio is, and if there are any protective measures that can be taken to reduce the risk. In addition, social, cultural, and political factors may also be considered. The outcome of this process is a recommendation for risk management.

Risk Management

Risk management is the decision process during which the results of the risk assessment are used to develop and analyze options for avoiding or minimizing risks of exposure to a given substance, taking into consideration political, social, cultural, economic, and technical aspects. The aim of this process is to define the best possible and feasible action(s). Risk assessment and management are distinct, though closely related, interactive processes: while risk assessment is a scientific, technical discipline, risk management is a sociopolitical decision-making process. Newer models of risk analysis have endeavored to develop a closer interlink between the two processes (see below).

The Process of Risk Assessment

Scientific information needed to conduct risk assessment includes qualitative and quantitative data on the toxicity of the agent in question, on the dose-response relation, as well as on the exposure (WHO 1999; Younes et al. 1999).

The process of collecting or extracting relevant data to be used for the risk assessment includes various steps which are tightly related to the problem formulation (the scientific question to be answered), the conceptual framework, and the definition of the evidence needs. The aforementioned considerations should always be clearly addressed in advance before the actual risk assessment begins. When the collection of the data has been completed, the risk assessment can be conducted.

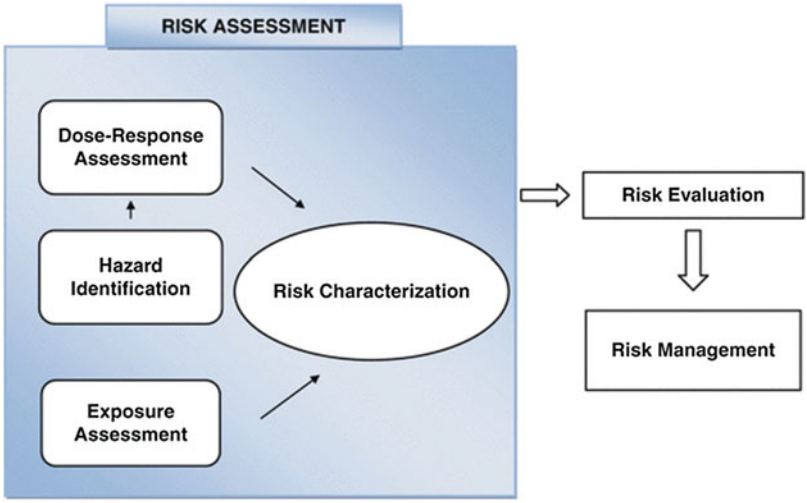

Fig. 1 Schematic presentation of the risk analysis process: Following risk assessment, with its four components, risk evaluation is conducted to allow for consideration of additional factors, such as socio-economic impacts, before risk management decisions are taken

Various paradigms have been developed to facilitate a systematic analysis of such complex data and, consequently, to allow for the development of a comprehensive estimation of potential risks. The most commonly used scheme worldwide is the one developed by the US National academy of Sciences (NAS) in 1983 (NRC 1983, 2009). It is currently in use by many regulatory agencies, though some variations of it are also applied, and more modern approaches have expanded on it to provide a better link between the processes of risk assessment, management, and communication. The NAS model divides the process of risk assessment into 4 distinct steps (Fig. 1).

Hazard Assessment

In order to better understand the important step of hazard assessment, it will be subcategorized below in two steps which altogether consist the process of assessing the hazardous properties of an agent.

Hazard Identification: Assessing the Potential to Cause Harm

It is worth noting that the terms "hazard" and "risk" are often used synonymously. This is incorrect. The term "hazard" describes the "potential to harm," that is, the principal ability of a given substance to exert a toxic effect (which, logically, will

only occur at a certain exposure level). Hazard is therefore an inherent characteristic of the agent in question. "Risk," by way of contrast, describes the probability that a harmful effect will, in fact, occur. Risk is the actual or potential danger posed by an existing or an expected exposure.

Hazard identification is the step during which all relevant data are analyzed that provide information to assess the inherent potential of an agent to exert harmful effects.

When the scientific question is relatively simple and can be addressed directly then a straightforward assessment can be conducted to reach an outcome following the steps described in this chapter.

In many assessments, however, questions may need to be subdivided to yield more directly answerable questions, and a weight of evidence assessment needs to be conducted.

The weight of evidence is comprised by three basic steps (see Fig. 2):

1. Assembling the evidence into lines of evidence of similar type which involves searching for and selecting evidence that is relevant for answering the question at hand, and deciding whether and how to group it into lines of evidence
2. Weighing the evidence which involves detailed evaluation and weighing of the evidence

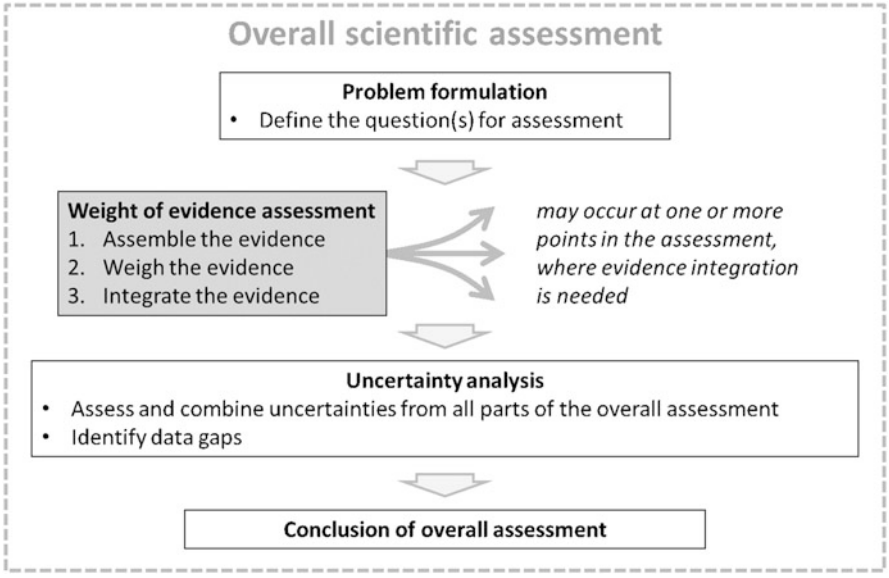

Fig. 2 Diagrammatic illustration of weight of evidence assessment as a 3-step process which may occur at one or more points in the course of a scientific assessment (EFSA 2017)

3. Integrating the evidence to arrive at conclusions, which involves weighing the relative support for possible answers to the question

It is important to note that reliability, relevance, and consistency of data are the three basic considerations for weighing evidence.

- Reliability is the extent to which the information is correct.
- Relevance is the contribution a piece or line of evidence would make to answer a specified question and how much could alter how decisions for a specific problem are taken, if the information comprising the evidence was fully reliable. This includes biological relevance.
- Consistency is the extent to which the contributions of different pieces or lines of evidence to answering the specified question are compatible.

Relevance and reliability may be considered in both the first and second steps since they are essential elements in order to identify the evidence to be used for the risk assessment but also when weighing the identified evidence.

Sources of data to be used for the hazard identification can be in vivo studies, in vitro studies, in silico (QSAR, read across, etc.), epidemiological studies, and control clinical studies on humans. A variety of studies are used to identify potential hazards of a chemical. More specifically, toxicokinetics considers how the body absorbs, distributes, metabolizes, and eliminates chemicals while toxicodynamics focus on the effects that chemicals have on the human body.

Especially, when assessing a chemical for potential adverse effects, analysis of a mode of action (MoA) and the development of an "adverse outcome pathway" (AOP) are currently used.

MoA is a biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data. A given agent may work by more than one MoA. For instance, when assessing a chemical for carcinogenic effects, the chemical might be involved in MoA both at different tumor sites as well as at the same site (Boobis et al. 2008).

The AOP approach provides a framework for organizing information at the chemical and biological level, allowing evidence from both in silico and in vitro studies to be rationally combined to fill gaps in knowledge concerning toxicological events. Fundamental to this new paradigm is a greater understanding of the mechanisms of toxicity and, in particular, where these mechanisms may be conserved across taxa, such as between model animals and related wild species. (Madden et al. 2014).

An AOP is defined as the information on the causal links between a molecular initiating event (MIE) which is the initial point of chemical-biological interaction within the organism that starts the pathway, intermediate definable key events (KEs) which make sense from a physiological and biochemical perspective and an adverse outcome (AO) of regulatory concern that is adverse at the individual level if discussing human health or population level if discussing environmental effects (see Fig. 3) (Ankley et al., 2010; Meek et al. 2014; OECD 2013).

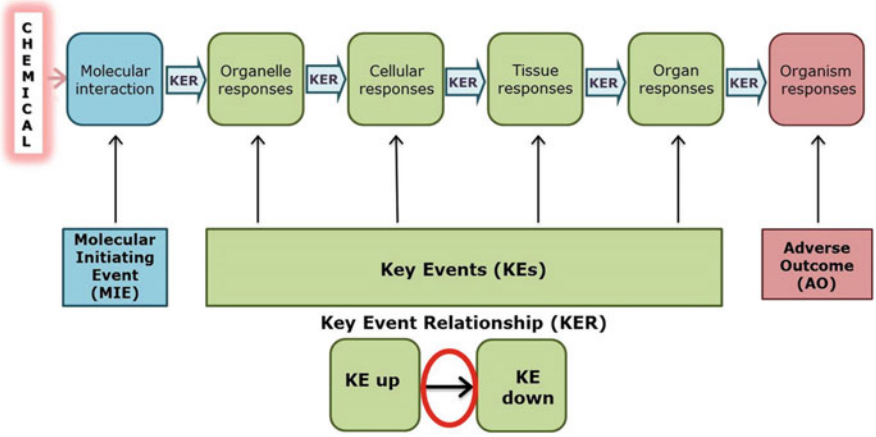

Fig. 3 An AOP consists of key events (KEs) and key events relationships (KERs) at different levels of biological organization starting from an initial interaction of a chemical with the biological system (molecular initiating event; MIE) through a sequence of KEs (cellular, tissue, organ, and organism) leading to an adverse outcome (AO) of regulatory relevance that represents overt adversity at either organism or population level. At sufficient concentrations and durations of exposure, KE up will trigger KE down, overcoming cell defense mechanisms and adaptation processes. (Anna Bal-Price et al. 2017)

Dose-Response Assessment: The Relation Between Exposure and Effect

The objective of hazard characterization is to document the dose-response relationship. Usually, as the dose increases, the measured response also increases. At low doses there may be no response. The adverse effect that occurs at the lowest dose is selected as the critical effect for risk assessment which serves for the derivation of a health-based guidance values. Different definitions can be found in bibliography (e.g., Acute Reference Dose (ARfD), Lowest or No-Observed–Adverse-Effect-Level (LOAEL/ NOAEL) or ideally BMD limit (BMDL))), but they all serve the same scope, which is to identify a reference point which will be consequently used for the derivation of a health based guidance value such as Margin Of Exposure (MOE) or Tolerable Daily or Weekly Intake (ADI/TDI). When this exercise is done, and the risk characterization is quantified, the risk assessor can conclude about the risk.

In the course of this step, a quantitative estimation of toxic effects, be it the severity of an observed outcome, such as the level of liver damage as evidenced by an increase in blood levels of liver-specific enzymes, or the frequency of occurrence of a yes-or-no outcome, such as cancer or even death, at different exposure levels is conducted. This allows for a characterization of potential toxic outcomes as a function of exposure or dose.

Table 1 shows the different reference points, health based guidance values, and ways to characterize the risk as they are used in risk assessment.

Table 1 Reference points and health based guidance values

Reference points (RPs)	Health based guidance value (HBGV)	Risk characterization
Benchmark response (BMR)	Acceptable daily intake (ADI)	Margin of exposure (MOE)
Lowest benchmark dose (BMDL)	Tolerable daily intake (TDI)	Risk characterization ratio (RC)
Benchmark dose (BMD)	Acute reference dose (ARfD)	Hazard quotient (HQ)
No observed (adverse) effect level (NO(A)EL)	Reference dose (RfD)	Margin of safety (MOS)
Lowest observed (adverse) effect level (LO(A)EL)	Derived-no-effect-level (DNEL)	Population at risk
No observed (adverse) effect concentration (NO(A)EC)	Derived-minimal-effect-kevel (DMEL)	
Lowest observed (adverse) effect concentration (LO(A)EC)	Population adjusted dose (PAD)	

Exposure Assessment

Exposure assessment encompasses the qualitative and/or quantitative determination of the level and frequency of exposure, potentially the lag time between subsequent exposures, the exposure media (air, drinking water, soil, recreational water, food), as well as the exposure route(s) (inhalation, ingestion, dermal absorption).

Uncertainty Analysis

A separate step is still needed to take account any uncertainties arising at all stages of the risk assessment. They should be addressed and described together with any data gaps. A separate step of uncertainty analysis is needed to take account of any uncertainties affecting the overall assessment. These are further categorized according to the source of uncertainty.

A. Uncertainties associated with assessment inputs which include:
    1) Ambiguity
    2) Accuracy and precision of the measures
    3) Sampling uncertainty
    4) Missing data within studies
    5) Missing studies
    6) Assumptions about inputs
    7) Statistical estimates
    8) Extrapolation uncertainty (i.e., limitations in external validity)
    9) Other uncertainties

B. Uncertainties associated with assessment methodology which include:
   1) Ambiguity
   2) Excluded factors
   3) Distributional assumptions
   4) Use of fixed values
   5) Relationship between parts of the assessment
   6) Evidence for the structure of the assessment
   7) Uncertainties relating to the process for dealing with evidence from the literature
   8) Expert judgment
   9) Calibration or validation with independent data
   10) Dependency between sources of uncertainty
   11) Other uncertainties

Risk Characterization: The Synthesis of Risk Information

The last step in risk assessment is risk characterization (see also ► Chap. 56, "Risk Characterization in Regulatory Toxicology"), which is a synthesis of all evaluated data and information. Strengths and weaknesses of the database must be clearly identified, methods, and criteria of all evaluations described, and the results of the evaluation of all data outlined. The outcome of risk characterization is the basis for developing strategies to avoid or, if this is not possible, to minimize the risk (risk management). Vulnerable groups, which are at particular risk due to higher exposure levels and/or an enhanced susceptibility, must be characterized in order for risk management decisions and actions to take their particular situation(s) into consideration.

The scheme described is a conceptual framework which should help in organizing all scientific data in a manner that allows a sequential, logical analysis. Other models/schemes have been developed, but the NAS paradigm is the most widely used till now. Individual steps of the process are more exhaustively described in other parts of this book.

Recent advances have been made to better link risk assessment with risk management. The US National Research Council recommended in 2009 that risk analysis should be divided in three phases. The first phase should cover problem formulation and scoping in order to better identify data needs and target risk assessment. The second phase should encompass the planning (stage 1) and conduct (stage 2) of risk assessment, pretty much following the NAS paradigm, but with an additional stage 3 to confirm the utility of the assessment. In this latter stage, questions to address include if the assessment had the attributes called for in the planning, if the assessment provides sufficient information to discriminate among risk management options, and if the assessment has been sufficiently peer-reviewed. Only then phase 3, risk management, actions can be evaluated and decided upon.

The Need for Harmonization

Despite the fact that the scientific data used for risk assessment purposes by different institutions are mostly identical for the same compound, they are often analyzed and treated differently and may result in different outcomes. For example, carcinogenic risk is characterized in the USA through a calculation of an exposure corresponding to a theoretical tumor incidence. In this context, dose extrapolation is conducted via different methods to very low levels, often below analytical detection limits. In this manner, exposures leading to a tumor incidence of, for example, 1 in 100,000 or 1 in 1,000,000 are calculated. Such methodologies are seldom used in Europe. Still, it is possible to compare the results of risk assessments conducted in different ways and to use performed data analysis to a certain degree, as long as the methodology, including all assumptions and uncertainties, is clearly outlined. It should be noted that there are recommendations to unify risk assessments for carcinogens and non-carcinogens, for example, in the 2009 report of the NRC.

At the international level, efforts are underway to harmonize, though not to standardize, risk assessment methods. In this context, the aim is to promote the understanding of different approaches to risk assessment, so that the results of such assessments conducted by a different institution can be understood by other institutions and eventually adapted to their specific needs. Thus, risk assessments can be utilized universally.

Risk assessment and the subsequent risk evaluation are the basis for regulatory decisions to manage risks. Regulatory measures are obviously different in different areas of regulation: In the case of pharmaceuticals, for example, the risk related to treatment must be put in relation to its therapeutic value. In the case of chemicals, it is important to estimate the potential direct exposure of workers in all areas (production, use, storage, and transport) and consumers, as well as the indirect exposure through various environmental media in order to reach regulatory decisions that would, indeed, eliminate or reduce to a minimum the exposure of the respective groups of the population.

Cross-References

- ▶ Risk Characterization in Regulatory Toxicology
- ▶ Risk Comparison in Toxicology
- ▶ Risk Evaluation in Regulatory Toxicology

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Part II

Tests and Methods

Monoclonal Antibodies: Discovery and Protein Engineering

7

Wibke Lembke and Mathias Locher

Contents

Abstract

In the past three decades, protein therapeutics have become an indispensable class of drugs approved by regulatory authorities for the treatment of a variety of diseases. Technological advances were key to allowing the generation of fully human therapeutic antibodies.

Protein engineering is a powerful tool to modify biophysical and biochemical properties, allowing control of posttranslational modifications (PTMs) and enhanced efficacy by modulating affinity. The mode-of-action can be tailored, best epitope selected, potential immunogenic regions eliminated, and pharmacokinetic properties optimized. This process helps to deliver molecules with superior efficacy and safety profiles, allowing the best protein therapeutic candidate to enter preclinical and clinical development. Detailed information on the protein

W. Lembke (✉)
Zurich, Switzerland
e-mail: wibke.lembke2@ucb.com

M. Locher
Locher Biotech Consulting GmbH, Kuessnacht am Rigi, Switzerland

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therapeutic characteristics needed within a particular drug development program can be generated, monitored, and regularly updated using high-performance modeling tools.

Keywords

Protein engineering · Antibody discovery · Antibody engineering · Protein therapeutics · Pharmacokinetics · Immunogenicity · Bispecific · Scaffolds · Antibody drug conjugates · Biologics · Half-life extension

Definitions

Protein therapeutic	Any protein-based drug.
Large molecules	The name originates from the fact that proteins are, other than chemically well-defined small molecules, comprised of a long amino acid chain folded into a three-dimensional structure and is usually produced in different isoforms.
Biologic	A biologic drug is produced from living organisms.
Monoclonal antibody	A monoclonal antibody derives from a single cell clone. The clone can be expanded and stored as a defined Master Cell Bank to produce the antibody in large quantities as a well-defined product.
Antibody fragments	Antibody fragments consist of part(s) of an antibody resulting in smaller functional molecules compared to a full antibody.
Bispecific	Bispecifics are engineered antibodies with the ability to bind to two distinct targets (different antigens or epitopes).
Alternative protein scaffolds	Protein therapeutics of a non-antibody type. They provide a robust, conserved structural framework and a variable region which can be engineered for specific recognition of a target.

Introduction

In the past three decades, protein therapeutics have become an indispensable class of drugs approved by regulatory authorities for the treatment of a variety of diseases like cardiovascular, respiratory, hematological, kidney, immunological, oncological, and orphan diseases. The first FDA-approved monoclonal antibody in 1986 was Orthoclone OKT3® for the prevention of organ transplant rejection. In the meantime, the number of protein therapeutics approved by the FDA has dramatically increased, especially within the last 10 years, reaching its peak in 2018, where 17 new biologic license applications (BLAs) were approved (Mullard 2020). Antibodies transitioned from being research tools towards effective therapeutics for the treatment of diseases, where millions of people are treated every year worldwide. Patients clearly benefit from protein therapeutics, particularly where no adequate treatment is available, as targets or mode of action is not druggable by small molecules.

Generally, protein therapeutics are very specific and selective for the target exhibiting only few off-target liabilities in contrast to small molecules, where off-target effects are often seen due to lower specificity. Hence, protein therapeutics such as monoclonal antibodies, antibody fragments, bi- or multispecifics, Fc-fusion proteins/fusion proteins, and antibody-drug conjugates (ADCs) (Fig. 1) became a substantial part of drug development pipelines.

Antibody therapeutics, compared to small molecules, are large in size with a molecular weight of an antibody being roughly 150 kDa. Due to the size, effective tissue or tumor penetration, and intracellular targeting, as well as crossing the blood–brain barrier can still be seen as limitations of the antibody-based therapeutics (Smith 2015). Antibody fragments, however, have the potential to penetrate tissues and tumors in a much more efficient way due to their smaller size, but exhibit a much shorter serum half-life compared to a full-size antibody.

The structural complexity of protein therapeutics is not only due to their size (primary amino acid sequence) but also their ability to form secondary, tertiary, and even quaternary structure.

An antibody consists of two heavy (H, 50 kDa) and two light (L, 25 kDa) chains. A heavy and a light chain associates through disulfide bonds and non-covalent interaction to form a heterodimer. Two heterodimers associate to complete the antibody, via the formation of disulfide bonds within the hinge region of the heavy chains.

Both heavy and light chains have constant (C) and variable (V) regions. The variable region, especially the CDRs (complementarity-determining regions), are key in mediating the target-antibody interaction (Fig. 1). Due to the bivalent nature of the antibody structure, an avidity effect can have a remarkable impact on the target binding kinetics.

Using antibody fragments such as single-chain variable fragments (scFvs) or Fabs (Fig. 1) allows the drug developer to play around with avidity and valency effects of the target binding interaction. Furthermore, the smaller size of such antibody fragments might allow binding to otherwise less accessible epitopes.

Even more complexity is introduced by the fact that proteins undergo posttranslational modifications (PTMs), which are heavily dependent on the expression system used. PTMs, such as glycosylation within the Fc part of an antibody, influences its stability and mediates important immune effector functions, underlining the importance of PTMs and its characterization in the final product.

Technological advances and discoveries made as well as experiences gained over the last years in the field of protein engineering have made the transformation from murine to chimeric to humanized to fully human antibody therapeutics possible (Fig. 2). The progress that was made towards fully human therapeutics allowed the immunogenicity risk to be reduced tremendously, as a human anti-mouse antibody (HAMA) response in patients treated with murine antibodies can be avoided. In contrast to small molecules, immunogenicity, namely the ability of a drug to induce an anti-drug antibody (ADA) response and thereby inducing an unwanted immune response in patients, is a unique feature of protein therapeutics and one of the biggest safety concerns.

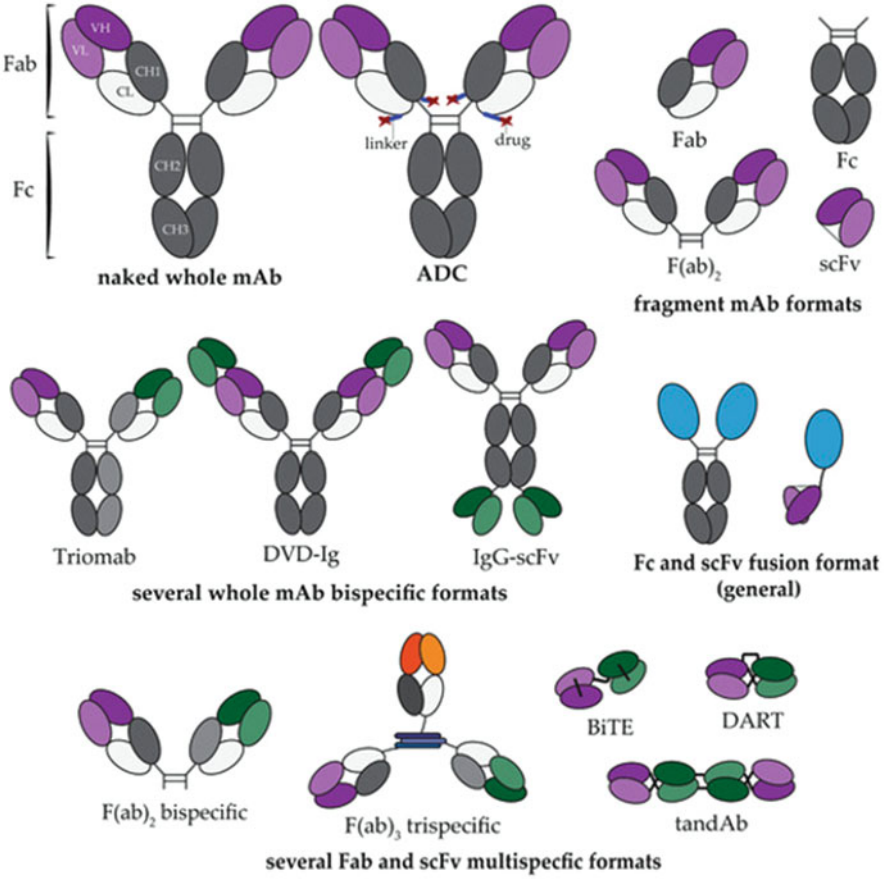

Fig. 1 Schematic representation of a whole monoclonal antibody (mAb), fragment mAb, and prominent fusion mAb formats for therapeutic use. A monoclonal antibody consists of two heavy (H) and two light (L) chains which are further divided into a constant (CH and CV) and a variable (VH and VL) regions which are covalently linked via disulfide bonds located in the hinge region (here shown as lines) forming its characteristic Y-structure. Based on papain digest, antibodies can be separated into a crystallizable (Fc) and antigen binding (Fab) fragments. Proteins fused to mAb fragments are depicted as blue ovals for a general representation; however, fusion proteins may vary in size and structure. Fragment formats include the Fc, Fab and F(ab)2, and single-chain variable (scFv) fragments. Further whole mAb formats include the antibody–drug conjugate (ADC), triomab, dual variable domain immunoglobulin (DVD-Ig), and immunoglobulin–scFv fusion (IgG-scFv). Multispecific fragment formats include the F(ab)2 bispecific, bispecific T-cell engager (BiTE), dual affinity retargeting molecule (DART), and tandem diabody (tandAb). (Sifniotis et al. 2019, **(CC BY)**)

To optimize an antibody-based therapeutic, one must understand how an antibody is constructed and the function of each part. Manipulation of the protein sequence and structure is known as protein engineering. Understanding the connection between sequence, structure, and function allows us to design protein therapeutics

by building-in desired properties. A variety of technologies are available to engineer proteins based on the therapeutic need. Nowadays, even machine learning can be applied to protein function optimization which will help to reveal unknown protein functions in the future and to uncover the relationship between protein sequence and its corresponding function (Yang et al. 2019).

Protein engineering is a powerful tool in the development of safe and efficacious next generation protein-based therapeutics, bringing more convenience to the patient.

Antibody Discovery

Technological advances were key to allowing the generation of fully human antibodies. As shown in Fig. 2, antibody therapeutics evolved from murine to fully human therapeutics over time. Replacing the constant regions of a murine antibody by the human counterpart can generate chimeric antibodies. One example for such a chimeric antibody is Rituxan®, an anti-CD20 antibody for the treatment of leukemia and lymphoma. Introducing a further protein engineering step to replace the murine framework regions in the variable domain of an antibody with human sequences results in a so-called humanized antibody. Examples of approved humanized antibodies are Avastin® and Zynbrita®. As the CDRs (complementarity-determining regions) in humanized antibodies are of nonhuman origin, they still bear a higher risk of unwanted immunogenicity compared to fully human antibodies (see section "De-risking the Immunogenic Potential of Protein Therapeutics by Protein Engineering").

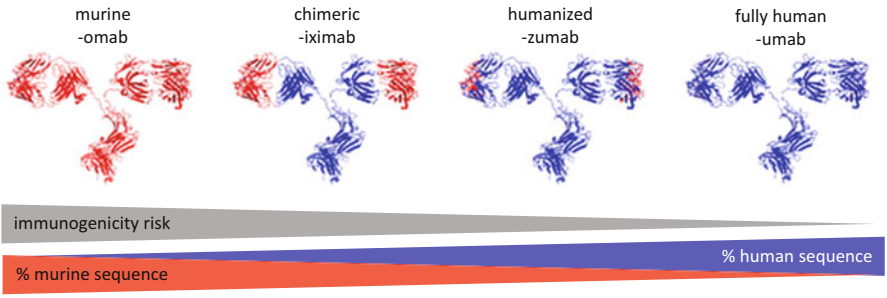

Fig. 2 The evolution of murine, chimeric (~70% human sequence), humanized (~90% human sequence), and fully human monoclonal antibodies through protein engineering to reduce the immunogenicity risk. Red and blue represents mouse and human sequences, respectively. The antigen-binding complementarity-determining regions (CDRs) are shown as sticks. Chimeric antibodies consist of a human constant region and a mouse variable region, whereas in a humanized format, only the hypervariable region is of murine origin and the framework within the variable region as well as the constant part is of human origin. The generic suffix of the international nonproprietary names (INN) for antibodies indicates its origin as shown in the figure. (Adapted from Leung et al. 2020, **(CC BY)**)

The first approved murine and chimeric antibodies were generated either using immunization or hybridoma technology. Both technologies were transferred successfully to human donors allowing the generation of fully human antibodies, but this is limited to a few indications, where human donors have developed a natural antibody response. For that reason, not all identified targets were druggable using this technology and protein therapeutics were limited to specific indications. In the 1990s, various companies have developed transgenic mice or rats, allowing the generation of human antibodies by in vivo immunization and hybridoma technology. These transgenic animals were endowed with the human antibody gene repertoire or parts of it.

To overcome the challenge that not all types of antigens are suitable for an immunization approach, in vitro selection technologies such as antibody phage display (Fig. 3), have been established (Frenzel et al. 2016). Phage display allows discovery of suitable candidates even when the target is highly conserved between species, toxic, unstable, a transmembrane protein or a protein with allosteric conformational changes. In addition, it broadens up the range of epitopes to include ones that are naturally suppressed by the immune response, thereby overcoming the limitations of immunological tolerance mechanisms. While the advances in technology allowed the use of antibodies for broader targets, oncology and hematology remain the most prevalent medical applications so far (Fig. 4b).

Using phage display technologies, large libraries containing more than 10^{10} different antibodies give access to an expansive antibody repertoire. Generally, different types of libraries are established: (1) immune libraries, constructed from V genes isolated from plasma cells derived from immunized donors (unrearranged V genes) and (2) naïve libraries generated from natural naïve human germ lines (rearranged V gene pools of nonimmunized individuals) (Frenzel et al. 2016). Humira®, one of world's best-selling drug, was the first approved phage-display-derived, fully human monoclonal antibody.

Antibody discovery is nowadays a fast process of 2–4 weeks with high success rate, typically yielding affinities in the low picomolar range. Even femtomolar binders are not uncommon.

Fc Engineering to Modulate the Mode of Action

Antibodies (also named immunoglobulins, Ig) can be divided into different classes and subclasses based on their sequence. The determination of the immunoglobulin G (IgG) subclasses, namely IgG1, IgG2, IgG3, and IgG4, is based on differences in the constant region of the heavy chain (CH), leading to structural variations in both the Fc part and the hinge region (Fig. 1).

The hinge region is a short sequence that links the Fab (fragment antigen binding) to the Fc (fragment crystallizable) region, where the heavy and light chains are covalently bound by forming disulfide bonds (Fig. 1). The effector function of the IgG subclasses is impacted by these structural differences.

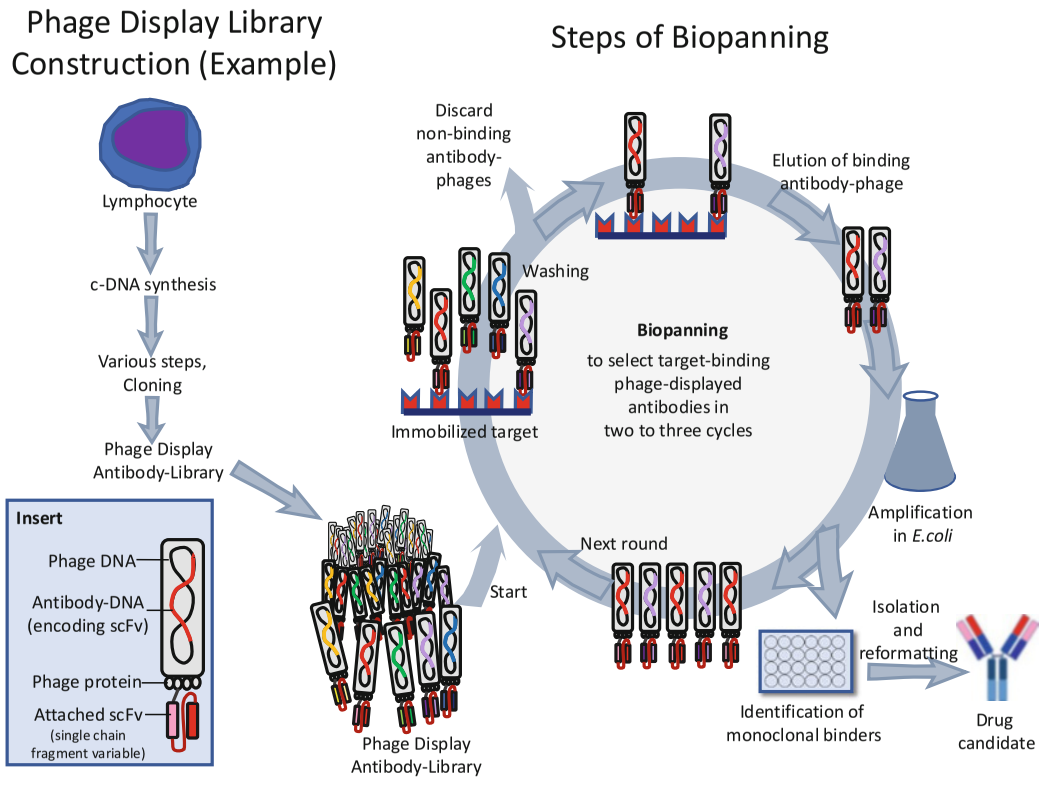

Fig. 3 Schematic representation of phage display technology for drug discovery including library generation, selection, screening, and reformatting. Characteristics of the antibody-like biophysical and biochemical properties, epitope and confirmation specificity, interspecies cross-reactivity, reduction of off-target binding, can be influenced during the selection process. Parameters, the protein engineering can play with to influence the outcoming antibody

The Fc part of the antibody brings in additional options for a protein engineer, due to the natural effector functions which can be modulated in tandem with the target specific interaction. The choice of the antibody subclass together with protein engineering approaches can impact the mode-of-action. Fc-mediated effector functions can thereby be either eliminated or even enhanced.

To date, the majority of the antibodies developed are of an IgG1 type with a kappa light chain and are produced almost exclusively using mammalian cells (Fig. 4c). IgG3 antibodies exhibit a long hinge region that is prone to proteolytic cleavage and is therefore not favorable for therapeutic use, whereas IgG4 antibodies on the other hand lack effector functions, which can be favorable for specific applications.

The ability of the Fc-part to bind to the neonatal Fc receptor (FcRn) is a key determinant in maintaining and prolonging long serum half-life of antibodies (Mackness et al. 2019). FcRn binding promotes antibody recycling, rescuing the protein from normal lysosomal degradation. The binding is strongly pH-dependent: high affinity binding is achieved at low pH, whereas under physiological pH conditions, no binding occurs, ensuring antibodies are returned to the cell surface rather than directed to a degradation pathway.

The average half-life of non-engineered IgG antibodies is around 21 days in humans. In contrast, the half-life of antibody fragments, proteins or peptides lacking a functional Fc is hours rather than days, as molecules below 50–60 kDa are subject to rapid renal clearance (Smith 2015). Thus, so-called Fc-fusions, in which an Fc is fused genetically to a protein of interest, have emerged to confer antibody-like properties on proteins and peptides of therapeutic interest (Levin et al. 2015).

Beside the influence on half-life by the Fc, important immune system effector functions can also be mediated by engagement of Fc gamma receptors (FcγR) and C1q binding (Saunders 2019; Corraliza-Gorjón 2017), namely antibody-dependent cell-mediated cytotoxicity (ADCC), complement-mediated cytotoxicity (CDC), and antibody-dependent cellular phagocytosis (ADCP) (for more details, see Fig. 5).

Fig. 3 (continued) characteristics, are: the library used in first place, the nature and immobilization strategy of the target antigen, as well as binding and washing conditions. Phage display technology is based on the presentation of proteins encoded in a library on the phage surface by fusion to a coating protein of the phage. Phage display libraries can be generated by cloning the relevant information into a phage background. Such libraries can contain more than 10^{10} different clones resulting in phages, expressing different proteins of interest on their cell surface. The information of the specific protein expressed on the surface is genetically encoded within the phage. The *in vitro* selection of binding proteins from libraries is called panning or biopanning. A panning circle contains the following steps: the antibody phage display library is incubated with an immobilized or soluble target. In a next step, nonbinding and nonspecific binding phage is removed by extensive and stringent washing. Subsequently, the bound antibody phage will be eluted and amplified by infection of *E. coli*. Such sublibrary will be used as new starting material for the next round until a significant enrichment of antigen-specific phage is achieved. Each sublibrary can be analyzed for the selected clones by screened for individual binders by ELISA. Promising candidates can be characterized by converting into appropriate formats for further testing

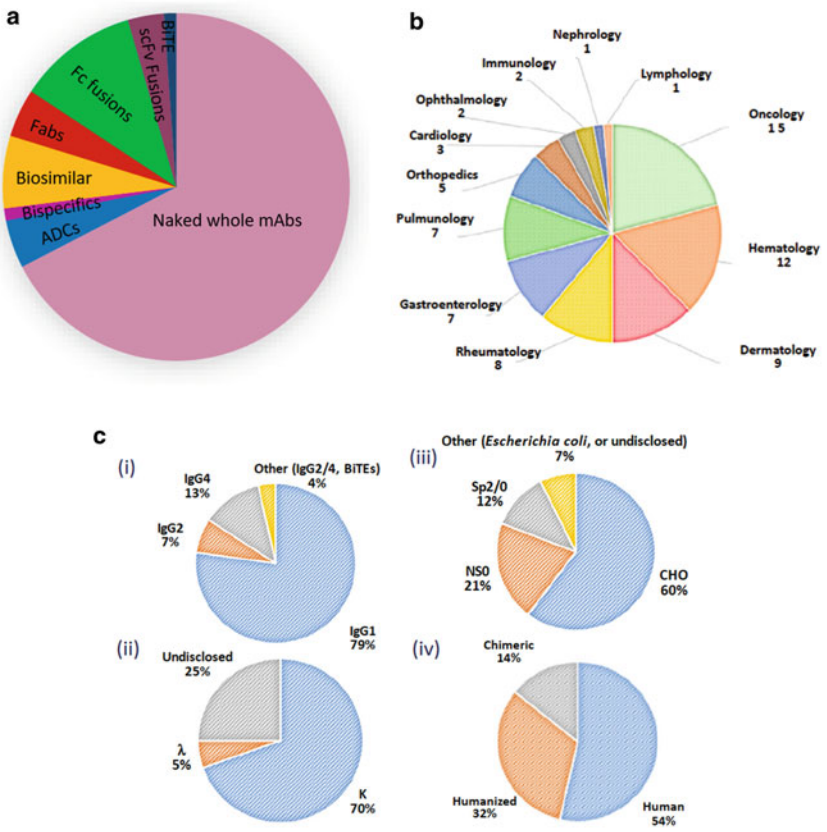

Fig. 4 (**a**) Approved therapeutic antibody formats as of 2018: pie chart of the proportions of therapeutic antibody formats approved for therapeutic use as of December 2018, IMGT®: 60 naked whole mAbs, 4 ADCs, 1 whole mAb bispecific, 4 Fabs, 10 Fc fusions, 3 scFv fusions, 1 scFv bispecific (BiTE), and 6 biosimilars. Adapted from Sifniotis et al. 2019 **(CC BY)**. (**b**) While mAbs are increasingly used for broader targets, oncology and hematology remain the most prevalent medical applications. (Grilo and Mantalaris 2019 with kind permission from Elsevier). (**c**) Inspection of the molecular structure of mAbs and production organisms of choice reveals the human nature of mAbs, which are mostly engineered on the IgG1 subclass with a kappa light chain and are produced almost exclusively using mammalian cells. (Grilo and Mantalaris 2019 with kind permission from Elsevier)

Glycosylation, in particular the N-linked glycan structure of the heavy chain at Asn297, plays an important role in effector function as this specific posttranslational modification influences the binding to FcγR (Zheng et al. 2011). Consequently, glycoengineering became an attractive way to specifically modulate Fc-effector function of antibody therapeutics.

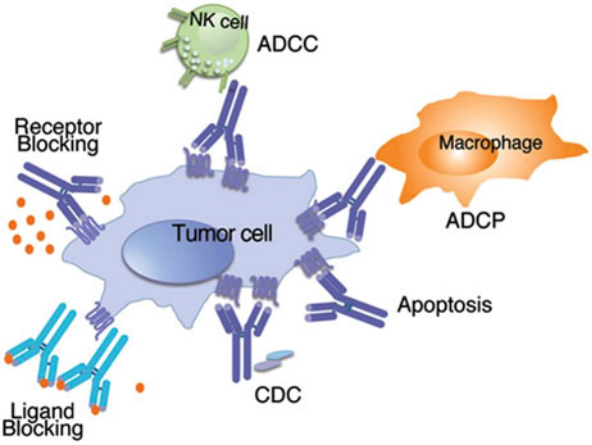

Fig. 5 Schematic representation of the mechanisms of action used by naked antibodies to inhibit tumor growth. Naked antibodies can inhibit tumor growth through effector functions such as ADCC (antibody-dependent cell-cytotoxicity) where the antibody bound to the tumor antigen is recognized by the natural killer (NK) cell and triggers cytotoxic activity; can also trigger antibody-dependent cell-phagocytosis (ADCP) when the antibody bound to the tumor antigen opsonizes the cell and activates phagocytic cells; the antibody can also fix complement after binding to the tumor cell and trigger complement-dependent cytotoxicity (CDC). Conversely, naked antibodies can kill the tumor cells by interfering with important signal pathways, either by binding to the ligand (Ligand Blocking) or by binding to the receptor (receptor blocking). In addition, they can trigger direct apoptosis after binding to an antigen on the tumor cell surface. (Corraliza-Gorjón et al. 2017 **(CC BY)**)

Engineering Half-Life of Protein Therapeutics

The desire to increase circulating half-life of protein therapeutics is based on the advantages associated with it. Direct results of a prolonged serum half-life include increased efficacy, reduced dose, and less frequent dosing regimen, which positively impact patient convenience.

As the average half-life of antibody therapeutics compared to other drugs is rather long and in a range of 2–3 weeks, half-life extension technologies focus on antibody fragments or scaffolds exhibiting a high systemic clearance usually induced by glomerular filtration in the kidney. To improve the PK of such protein therapeutics, PEGylation, glycoengineering, and engineering of Fc effector functions are instruments in the toolbox of the drug developer to extend the serum half-life (Fig. 6). The most common and so far most commercially successful approach to prolong the half-life of protein therapeutics has been PEGylation. PEGylation describes the covalent attachment of polyethylene glycol (PEG) chains to a protein therapeutic. However, there is growing concern about the safety of PEGylation, as this modification may lead to hypersensitivity reactions, ADA formation, and PEG bioaccumulation (Witteloostuijn et al. 2016). Alternatively, albumin conjugation and Fc-fusions are well-established methods to reach an antibody-like half-life (Fig. 6). Such therapeutics

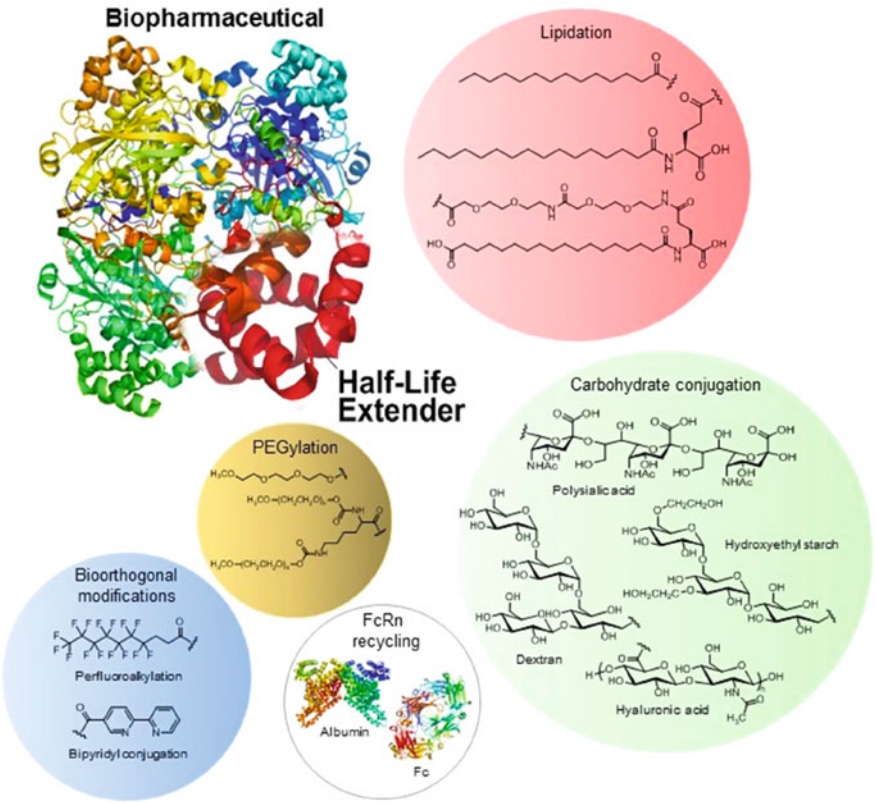

Fig. 6 Half-life extension possibilities for protein therapeutics. Native peptides and proteins suffer from an inadequate pharmacokinetic profile, and their outstanding pharmacological potential can only be realized if this issue is addressed during drug development. To overcome this challenge, a variety of half-life extension techniques relying on covalent chemical modification have been developed. These methods include PEGylation, fusion to unstructured polypeptide-based PEG mimetics, conjugation of large polysaccharides, native-like glycosylation, lipidation, fusion to albumin or the Fc domain of IgG, and derivatization with bio-orthogonal moieties that direct self-assembly. (Witteloostuijn et al. 2016 with kind permission from John Wiley and Sons)

are easy to generate by genetically linking the protein therapeutic via a short amino acid linker to albumin or an Fc-part. In addition to the anticipated positive effect on the PK, Fc-fusions can simplify the purification process of the protein drug.

De-risking the Immunogenic Potential of Protein Therapeutics by Protein Engineering

Immunogenicity, the development of antibodies against therapeutic proteins (ADA = anti-drug antibodies), is one of the challenging liabilities of protein therapeutics. The risk associated with a treatment-induced immune response needs

to be carefully considered during drug development. Immunogenicity risk assessment has become an integral part that goes beyond the molecular design phase to development and post-marketing evaluation that is required by regulatory authorities.

The immunogenicity rate observed in the early days of protein therapeutics has been dramatically reduced by evolving from murine to chimeric or humanized to fully human protein therapeutics (Fig. 2). However, even fully human therapeutics may be able to induce an immune response. Until now, a lot of effort has been made to generate and develop comprehensive and accurate in silico, in vitro, ex vitro, and in vivo immunogenicity prediction tools.

Immunogenicity de-risking strategies are routinely used and guide the de-immunization of drug candidates by knowledge-based protein engineering, and where possible B-cell or T-cell epitopes are eliminated. Not only the primary sequence, also patient-, product- (e.g., manufacturing procedure), and treatment-related (e.g., route of administration) factors are known to influence the immunogenic potential of a protein therapeutic (see Fig. 7) and are optimized to mitigate the overall risk within the drug development program.

An unwanted immunogenicity response by the induction of ADAs can alter the pharmacokinetic and pharmacodynamic properties of a drug raising a concern for both safety and efficacy. ADAs usually result in an accelerated drug clearance and may induce hypersensitivity responses leading to severe safety issues. In addition, the appearance of neutralizing antibodies can lead to a dramatic impairment of treatment efficacy, beside loss in exposure.

A Two-in-One Approach: Bispecific Protein Therapeutics

Using protein engineering technologies, antibodies or antibody fragments can be designed in a way that a second specificity or even multiple specificities can be combined in one therapeutic molecule. Making use of these possibilities has expanded the therapeutic treatment opportunities for diseases where more than one target or pathway is critical (Smith 2015). One example for antibody-based bispecific molecules is the Duobody platform, which is inspired by the naturally occurring Fab arm exchange observed for IgG4s. In short, the generation of bispecific molecules includes three steps. In a first step, two IgG1s, each containing single matched mutations in the third constant (C_H3) domain, are produced separately using standard mammalian recombinant cell lines. After production and purification of the two IgG1 antibodies, they are recombined using favorable conditions resulting in a bispecific antibody product with a very high yield (Labrijn et al. 2013).

Another example is the knob-into-hole technology, where heterodimerization of the heavy chains is promoted by introducing mutations: a “knob” and a “hole” are engineered into the two heavy chains of different antibodies to favor the heterodimerization process (Xu et al. 2015).

Bispecific formats allow for introducing new mode of actions. The Bispecific T-cell Engagers (BiTEs®) platform is a good example, where the single-chain variable

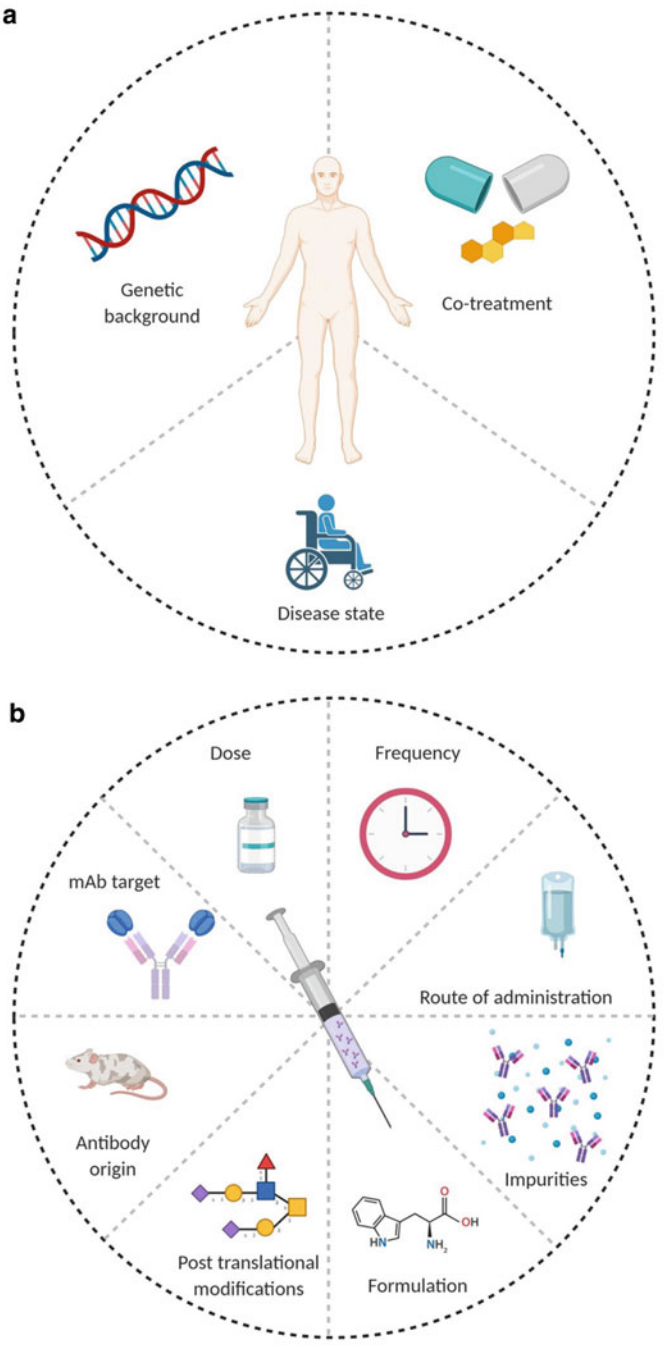

Fig. 7 The possible causes of anti-drug antibodies are either patient related (**a**) or drug related (**b**). Drug-related risk factors such as origin, posttranslational modifications, as well as dose and frequency can be positively influenced by protein engineering technologies. (Vaisman-Mentesh et al. 2020; © 2020 Vaisman-Mentesh, Gutierrez-Gonzalez, DeKosky and Wine, **(CC BY)**)

(scFv) fragments of two antibodies are fused to each other using short linkers (Fig. 1). In the case of the 2014 approved BiTE® Blincyto®, an anti-CD3 scFv is combined with an CD19-binding scFv. This results in therapeutic molecule that links the patients T cells via CD3 binding to the tumor-associated antigen (TAA) CD19, resulting in effective tumor cell killing.

Bispecifics bear the potential to overcome the blood–brain barrier hurdle or membranes by targeting with one specificity a cargo or transporter molecule. This will open the space for protein therapeutics towards intracellular targets or targets in the brain.

Scaffolds: The Alternative Protein Therapeutics

Alternative scaffolds complement the antibody derivatives as possible protein therapeutics. Notably, typical properties for which the scaffolds were earlier advertised, such as small size, high-level recombinant expression, and advantageous biophysical properties, have become less relevant, as antibody engineering has advanced into the field of single-domain antibodies (Gebauer and Skerra 2019).

Computation protein design (CPD) has the unique ability to design functional scaffolds de novo and to create artificial proteins bearing the desired function. The majority of scaffolds worked on are inspired by naturally occurring domains that are involved in protein–protein interactions (Ernst and Plückthun 2017).

Antibody-Drug Conjugates (ADC): Combining the Best of Two Worlds

ADCs are generated to combine the best properties of both worlds, the small and large molecules, for the treatment of cancer. The beauty of this technology is the use of specific delivery by the antibody combined with a cytotoxic drug, reducing toxicity and improving efficacy compared to a systemic delivery of conventional chemotherapies. The first generation of ADCs in the 1990s failed due to various reasons including: immunogenicity (antibodies used were of murine or chimeric nature) and linker instability. In addition, no efficacious quantity of drug could be delivered into the tumor cells, for reasons including low potency of the payload, low antibody-drug-ratio and antigen density on the tumor cell. Within the second generation of ADCs, fully human antibody conjugates with stabilized linkers have successfully delivered potent chemotherapeutic and radioactive agents to specifically kill tumor cells and thereby overcome the liability of chemotherapy and radiation to distinguish between healthy and tumor cells (Leung 2020). To date, four ADCs are approved for the treatment of cancer including Kadcyla® and Adcetris®.

While engineering a suitable ADC, carefully consideration should be given to find a selective target, an antibody exhibiting suitable binding properties towards the

target, type, and stability of the linker, potency of the payload, the conjugation method, and the drug-to-antibody ratio (Smith 2015).

To avoid systemic toxic release due to linker instability, the ideal situation would look as follows (Leung 2020): the payload is inactive while stably linked to the antibody in circulation, but upon target-dependent internalization, the cytotoxic drug is rapidly released after lysosomal degradation of either the linker or the antibody.

Antibodies can be labelled using unspecific technologies like labelling of lysine and cysteine residues or site-specific, which allows for a better-controlled process in terms of homogeneity of the resulting drug product. Engineering approaches to site-specific conjugation include substitution or addition of accessible cysteines or incorporation of non-native amino acids (Smith 2015). In addition, enzymatic methods have been established, where site-specific sequences have been engineered into the antibody to facilitate site-specific conjugation (Leung 2020).

To aim for less systemic toxicity, other approaches than ADCs are currently tested including antibody-directed enzyme therapy (ADEPT), antibody-cytokine fusion proteins or immunocytokines, immunotoxins, and radioimmunoconjugates (Smith 2015).

Cross-References

- ▶ Biomolecules Versus Smaller Chemicals in Toxicology: ICH, EU, and US Recommendations
- ▶ Biopharmaceuticals: CMC Development "Points to Consider" from a Regulatory Perspective
- ▶ Examination of Organ Toxicity
- ▶ Toxicological Aspects in the Regulation of Gene Therapy Medicinal Products

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Characterization of Physicochemical Parameters in Toxicology

8

Mathias Locher

Contents

Abstract

Toxicological tests are only meaningful and interpretable, when the chemical composition and the physicochemical properties of the applied substances are known. These properties determine to a large extent the behavior of a substance in the environment and in the living organism. Thus, knowledge of physicochemical properties is important for the development of therapeutic drugs as well as for the risk assessment of all chemicals.

Keywords

Solubility · Permeability · Distribution · Artificial intelligence · Analytical methods · Impurities · Content

M. Locher (✉)
Locher Biotech Consulting GmbH, Kuessnacht am Rigi, Switzerland
e-mail: mlocher56@icloud.com

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Introduction

In most fields of toxicology, including industrial chemicals, environmental pollutants, or pharmaceutical products, the chemical's physicochemical features are of great relevance, since they determine solubility, volatility, absorption, and toxicokinetics. In environmental toxicology, knowledge of physicochemical features allows to anticipate, whether a substance will be transported in the environmental media air, water, and soil. The Organisation for Economic Co-operation and Development (OECD) publishes international guidelines for testing of chemicals (OECD 1 2020) and environmental pollutants (OECD 2 2020). Guidelines for pharmaceuticals are published by the International Committee of Harmonization (ICH 2020) for small chemical drugs as well as biopharmaceuticals. We focus here on some aspects that are relevant for pharmaceutical drug development.

A drug-like chemical molecule is not only characterized by its binding to a target like a receptor or enzyme. A chemical molecule has to be signed for more, for the optimal biological activity, for a broad therapeutic window, to be optimally formulated, and last but not least for optimal absorption, distribution, metabolism, and excretion (ADME) behavior. All these parameters are finally driven by the physicochemical features of a chemical molecule. Therefore, assessing the physicochemical parameters of a pharmaceutical (test article) early on in hit and lead molecule screening is essential for the success.

Physicochemical Properties and Bioavailability

Physicochemical properties, like solubility and coefficient of distribution (octanol/water at pH 7.4), combined with particle size or crystal structure, usually influence and correlate with absorption, distribution, metabolism, and excretion (ADME) of drugs (Krämer and Wunderli-Allenspach 2001). In-depth knowledge of these basic physicochemical characteristics of a drug substance are important for the characterization of chemicals as well as for the development of innovative and ideal drug formulations and to optimize bioavailability of drugs. Figure 1 shows some simple methods to study physicochemical properties.

With regard to physicochemical properties, solubility and coefficient of distribution of a drug substance are mainly of interest for the toxicologist. The paramount of a toxicological investigation is to show adverse effects in order to be able to estimate the risk associated with human (or environmental) exposure to the substance. Therefore, the maximal dose, given in a toxicity test, and the resulting systemic exposure (measured in plasma or serum) should be as high as possible.

The systemic exposure is a delicate mixture of a series of events: the extent of absorption, extent of distribution, extent of metabolism, and extent of excretion. While of course drug molecules can be substrates of cellular transport systems (not discussed here), absorption, distribution, and accessibility for metabolism are influenced by the physicochemical properties like solubility and coefficient of distribution of the drug molecules. For the absorption of an orally administered

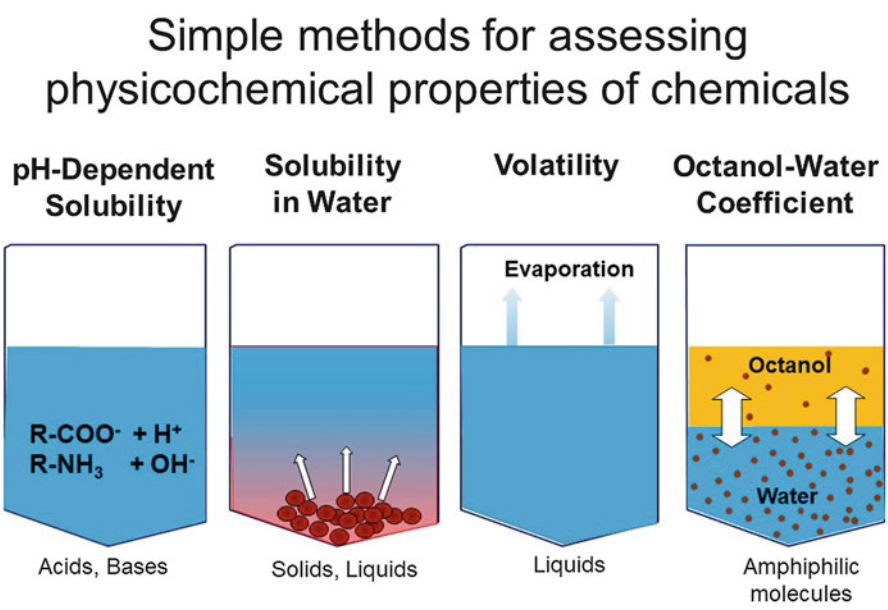

Fig. 1 Simple methods for assessing the physicochemical properties of chemicals

encapsulated powder or an administered suspension, the drug substance has to be solubilized in advance in the gastrointestinal tract. This sometimes rate-limiting step could be circumvented by the intravenous route, but for the practicability of a daily administration and for the convenience of the patients, an oral formulation for a therapeutic agent is highly preferable. Therefore – if possible – the oral administration of a solution to the animal species (e.g., rat) is not only the most simple approach; an oral solution usually guarantees high plasma concentrations (*Cmax*) and high systemic exposure (*AUC*).

Usually solubility is tested in a series of organic solvents. For the toxicologist, solubility in dimethylsulfoxide (DMSO) is important because this solvent is used to solubilize molecules to be tested in the Ames test. Of course, in addition, substantial knowledge should be available on the solubility of the test compound in buffer systems used for i.v. or oral administration to animals (e.g., rodents, dogs, monkeys). In pharmaceutical industry, the close interaction of the toxicologist with the chemist and pharmacist sets the basis of a straightforward risk assessment process.

To ensure the reliability of a toxicological study, the quantitative determination of the test article in the test solution is a must. Usually specific HPLC-based techniques like HPLC-UV or HPLC-MS are used for small molecule drugs.

For biotherapeutics like monoclonal antibodies (mAbs) or protein replacement therapies, partly other measurements and methods are of importance compared to small molecules (Swami and Shahiwala 2013). Proteins usually are not given via the oral route. The main administration routes for proteins in toxicology are either the intravenous or the subcutaneous route. Solubility and stability of the protein is of

great importance especially to prepare highly concentrated subcutaneous formulations (>100 mg/ml) for mAb toxicity testing. Aggregation of the protein has to be prevented; therefore, a series of buffer systems should have been tested before starting the toxicity testing of proteins. Quantitative determinations of the specific protein concentration in the solution, and of levels in plasma/serum, are still done by immunoassay techniques.

At present, many pharmaceutical companies face challenges in their drug development programs because of increased costs and reduced efficiency. Artificial intelligence (AI), machine learning (ML), and deep learning (DL) combined with new experimental technologies are expected to make the hunt for new pharmaceuticals cheaper and more efficient. One example is the use of AI/ML in drug screening. With the help of AI, a virtual compound library of several billion of molecules can be screened within a few days compared to several months when doing the traditional screening process (Voosen 2017; Baig et al. 2016).

AI tools are used in multiple aspects within the drug discovery process for predicting physical properties (Andrysek 2003; Lynch and Bothwell 2007), bioactivity, and toxicity (LeCun et al. 2015; Dixon et al. 2016; Wu et al. 2018) of a potential drug, to structure predictions. AI-based structure predictions only take a few hours to a few days compared to months or even years for the traditional experimental structural biology methods.

Identification, Content, and Stability of the Test Article

The test article tested in vitro or in vivo toxicology studies should be comparable to the test article used for clinical studies in humans. Therefore, information on the identity, the content, and the storage as well the benchtop stability of the test article are important information for the toxicologist and should be available before testing.

Testing the identity primarily tells the toxicologist if the test article has still the quality required for testing. As mentioned above, it is important to measure the concentration in the test solution to calculate the exact dose administered and to exclude that there is precipitation or adsorption of the test article to glass or plastic vessels used for the preparation of the test solution which could invalidate the toxicological study.

Methods

Methods to assess the identity of the test article should be able to discriminate the test article from structural similar molecules, i.e., the analytical methods should be specific (e.g., IR spectroscopy and mass spectroscopy). To identify a test article only by a single HPLC method is not acceptable. A second chromatographic method using a different separation or detection technique is necessary to ensure the identity of a molecule.

If the test article is an enantiomer, the method used to describe the identity of the test article should be able to discriminate between the enantiomers.

Compared to small molecules, the biotherapeutics (mAbs and other proteins) are complex molecules and only in rare cases exist as a single unique molecule. Proteins produced by expression in mammalian cells or bacteria usually exist in different isoforms, and their glycosylation pattern usually varies. These isoforms may have different pharmacokinetics, binding affinity, and bioactivity (European Medicines Agency 2007). Independent of the complexity of applied protein therapeutics, the test article tested in toxicology studies has to meet the specifications (melting temperature, SDS-page, molecular weight, glycosylation pattern, binding, bioactivity) of the material produced for human use.

Immunogenicity is a very special event following the administration of proteins to animals or humans. The resulting antidrug antibodies (ADAs) may influence not only bioactivity but also clearance of the protein. Therefore, it is necessary to assess immunogenicity using respective assays that not only measure ADAs as such but can also discriminate between neutralizing and non-neutralizing ADAs, which is important for the further toxicological testing strategy (Jefferis 2011).

Inhalative Toxicology

Inhalative toxicological studies ask for high analytical demands. The test article will be delivered as gas, aerosol, or powder that is aspirated by the test animals. Therefore, in addition to the usual analytical control of the test article, the particle size of the aerosol or powder as well as their homogeneous distribution within the experimental system has to be assessed.

Impurities and Content

The test article already used in early safety studies (safety pharmacology and short-term toxicology) should be comparable to the test article later tested in clinical studies and finally reaching market approval. Therefore, it is important that the content of the test article and the impurity profile – the specifications – of the early available drug substance meet the specifications of the drug product marketed later. At the beginning of drug development process, test article specifications should not be too tight; on the other hand, a high level of impurities may negatively influence results of toxicology studies, e.g., the Ames test. Specifications of the test article batches tested in safety relevant studies have to be listed in the regulatory documents and have to be compared with the specification of the batches used in clinical trials (Fig. 2).

The impurity profile is an important characteristic data set for the drug substance as well as the drug product and is related to the synthesis/production process of the test article. A major change in the impurity profile of a marketed product, e.g.,

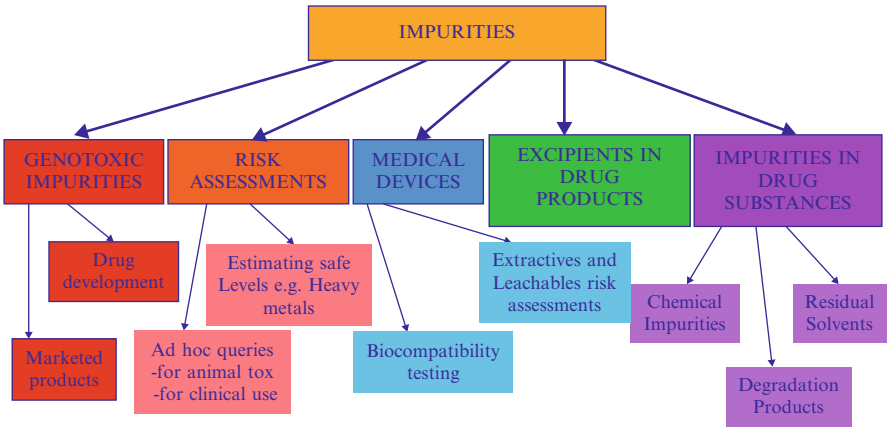

Fig. 2 Impurities and their importance in drug development

Fig. 3 Safety assessment – it's all about risk assessment

because of a process change, requires a new safety assessment that may include preclinical and clinical studies (Fig. 3).

For biotherapeutics aggregates, viral contaminations and host cell proteins are a major source for impurities. Therefore, a harvest and downstream process (purification process) has to be established using methods to separate the impurities from the products and with special procedures for virus inactivation.

Today, for small molecules, HPLC-MS/MS is the method of choice to quantify the test article and the impurities. For protein drugs, immunoassays (ELISA) are usually used to quantify the test article, and chromatographic, electrophoretic, or PCR methods are used to quantify impurities. But protein drugs have to be characterized further. In addition to the content, the bioactivity of the test article measured in a validated cell-based assay is usually required to characterize the test article and

to ensure the comparability of test article used in preclinical and clinical drug development with the marketed product.

Guidelines

For drug development, the International Conference on Harmonization (ICH) has published a series of guidelines for all aspects and phases (e.g., quality, efficacy, safety) of drug development (http://www.ich.org/products/guidelines.html).

Information on requirements to assess the quality (e.g., stability, impurities, specifications, analytical validation) can be found in the quality guidelines Q1–Q11. All regulatory requirements with regard to drug safety are summarized in the safety guidelines S1–S10. A special notice has to be given to the guideline S6. This guideline is only valid for the safety assessment of biotherapeutics.

It is well known that toxicological as well as safety pharmacological studies have to be performed according to the regulations of GLP. Therefore, the analytical methods used to characterize the test article have to be validated, and the analysis has to be performed accordingly. If analytical investigations are not performed accordingly, this has to be described and explained. In the USA, GLP regulations are described in the "21 CFR 58 – Good Laboratory Practice Regulations."

Cross-References

- ▶ Biomolecules Versus Smaller Chemicals in Toxicology: ICH, EU, and US Recommendations
- ▶ Computer-Based Prediction Models in Regulatory Toxicology
- ▶ Importance of Physicochemical and Physical Properties for Toxicological Risk Assessment
- ▶ Monoclonal Antibodies: Discovery and Protein Engineering
- ▶ Principles of Analytical Chemistry for Toxicology
- ▶ Toxicokinetic Tests

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Examination of Acute and Repeated-Dose Toxicity

9

Volker Mostert, Karl Georg Heimann, and Kevin Doughty

Contents

Abstract

Determination of the toxicological profile is necessary for hazard identification and hazard characterization for human health. Acute studies mainly serve the purpose of hazard identification for the classification and labelling of substances

V. Mostert (✉)
extera, Langenfeld (Rhld.), Germany
e-mail: vmostert@extera-vm.com

K. G. Heimann
Expert Regulatory Toxicology, Köln, Germany

K. Doughty
Registration Department, Bayer CropScience, Monheim, Germany
e-mail: kevin.doughty@bayer.com

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or mixtures. Repeated-dose toxicity studies using subacute, subchronic, or chronic exposure durations are used for hazard characterization. Such studies deliver the point of departure for setting health-based reference values, e.g., acute reference dose (ARfD), acceptable operator exposure level (AOEL), or acceptable daily intake (ADI).

Keywords

Toxicity study · Single dose · Repeated dose · Study protocol · LD50 · Acute toxic class · NOAEL

Introduction

Aim and Study Protocol

Toxicity studies have to be performed with chemicals and mixtures subject to chemical legislation or regulation (e.g., pharmaceuticals, pesticides, industrial chemicals, biocides). Before starting a study, a study protocol has to be issued according to accepted testing guidelines e.g., OECD (see references), European Union, US EPA, which exactly lays out the aim and methods of the investigation in detail. The methods have to be fixed by standard operating procedures (SOPs) to reduce as much as possible the systematic error rate and to ensure the transparency of the procedures and the resulting data (Good Laboratory Practice, GLP). The basic study types are shown schematically in Fig. 1. Acute studies involve administration of a single oral dose, 4 h inhalation exposure, or 24 h of dermal exposure.

Repeated-dose studies are conducted either by continuous exposure via feed or drinking water (oral), daily gavage administration (oral), or daily exposure via inhalation or dermal contact for 6 h/day.

Investigated Parameters

Acute toxicity studies aim at the identification of lethal doses or concentrations for the purpose of classification and labelling. Therefore, only a limited set of parameters is typically investigated: survival, clinical signs of toxicity, weekly body weight, and gross necropsy.

In repeated-dose toxicity studies, body weight development, feed consumption, detailed clinical observations (including functional observational battery), hematology, clinical chemistry, urinalysis, gross pathology (including organ weights), and microscopic pathology.

Where applicable, relevant statistical methods have to be employed to inform on the statistical significance of any treatment-related changes that are observed.

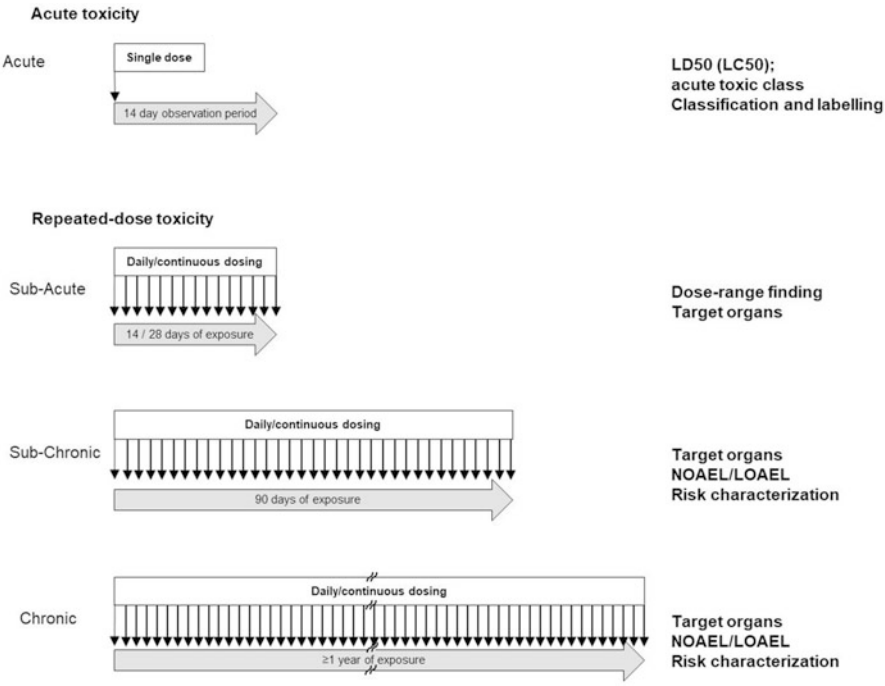

Fig. 1 Type and aim of the studies (schematic)

Reporting Requirements

The following experimental conditions have to be recorded: selection of an accepted species including the strain, starting weight/age, food/water quality and source, quality of substance (purity grade, homogeneity, stability in the application medium), temperature, air humidity/exchange/pressure and duration of lightning in the stall, size of cages/inhalation chambers, litter, hygiene management, use of validated methods and appropriate material, as well as follow-up of historical control data. The results have to be listed in the report as individual values and deposited as raw data – signed and dated – in an archive certified by GLP. Study protocol, test procedure, as well as the transfer of the results to the final report have to be proofed and certified by a designated and independent quality assurance unit. According to animal welfare regulations, animal studies which are not requested by authorities are subject to approval; *in-vitro* studies are notifiable only.

Acute Toxicity

The aim of acute toxicity evaluation is to estimate a dose or concentration that would lead to an expected 50% mortality of the exposed animals.

The main application routes are oral, dermal, as well as inhalation which are relevant routes of human exposure. There are further application routes like intraperitoneal or intravenous application that can be relevant for medical research. The further process is comparable. The single oral application is carried out as a bolus application (gavage/capsule) using 3–5 animals/dose/sex. Rats are the preferred species for the oral route of exposure.

Dermal application is performed moistened or as a pasty related to the physical condition (liquid/solid). The skin is treated under semi-occlusive coverage over 24 h. Rats or rabbits are the preferred species for the dermal route of exposure.

Testing via inhalation may employ exposure to dusts, liquid aerosols, or gases/vapors, depending on the physical properties of the test substance. Rats are typically used for acute inhalation studies. To determine the hazard/risk potential, the "nose-only" system is preferred where the animals are exposed exclusively via the respiratory tract (nose/mouth) over 4 h. This ensures that potential effects can be reliably ascribed to the intended application route and not, e.g., to oral ingestion of test material deposited on the coat from grooming behavior. After the single application, a 14-day post-application observation period follows which has to be enlarged if symptoms still continue. During this period progressing symptoms are protocolled related to intensity and start/end time in minutes/hours/days. Time of death of dead/moribund killed animals is protocolled in addition. Thus, conclusions can be made about symptoms and death related to the latency. Short latency periods hint to a substance, longer-lasting periods to an accumulated impact. At the end of the observation period, surviving animals are anesthetized, dissected, and assessed macroscopically (anatomic-pathological; dead animals directly). As further parameter, body weight has to be determined before treatment, weekly post-application, and before section. Based on the results (type, intensity, duration of symptoms, body weight gain), the acute toxic profile of a substance can be gained. Based on the number of dead animals/groups, the LD50 (median lethal dose, i.e., dose where 50% of the animals die) can be calculated according to Litchfield and Wilcoxon (Table 1), for example.

Acute Toxic Class Method

The Acute Toxic Class (ATC) Method (OECD guideline 423, see Reference) has largely replaced the conventional LD50 testing shown in Table 1. This method employs the use of fewer animals than the conventional method. Depending on the number of humanely killed or dead animals, the test procedure follows the indicated arrows. The scheme in Fig. 2 shows the procedure when the highest dose is chosen as starting point. This is appropriate if low toxicity of the substance is expected, e.g., from structurally related substances with known toxicity. Selection of the correct starting dose is essential for using the minimum possible number of animals.

Instead of distinct LD50/LC50 values, ATCs are obtained which are directly related to existing national/international classification systems (WHO, EU). The ATC method allows the derivation of LD50/LC50 cut-off values that can be used as surrogates for defined LD50/LC50 values.

Table 1 Dose response and median lethal dose (calculated according to Litchfield and Wilcoxon 1949)

Dose (mg/kg bw)	Toxicological results[a]	Duration of clinical signs	Time of death	Mortality (%)
Males				
50	0/0/5	–	–	0
100	0/0/5	–	–	0
150	0/5/5	52 m–2 day	–	0
170	0/5/5	2 h–2 day	–	0
200	3/5/5	2 h 15 m–1 day	3 h 45 m–1 day	60
300	1/10/10	2 h 30 m–2 day	5 h 30 m	10
1000	5/5/5	9 m–1 day	48 m–1 day	100
170 mg/kg bw < LD50 < 200 mg/kg bw				
Females				
50	0/0/5	–	–	0
100	0/2/5	2 h 45 m–1 day	–	0
150	2/5/5	51 m–2 day	5 h 45 m–1 day	40
170	0/2/5	3 h 45 m–1 day	–	0
200	4/5/5	2 h 15 m–1 day	3 h 30 m–1 day	80
300	4/10/10	29 m–1 day	2 h–1 day	40
1000	5/5/5	13 m–4 h 15 m	2 h 15 m–4 h 15 m	100
150 mg/kg bw < LD50 < 200 mg/kg bw				

[a]No. of dead animals/No. of animals with symptoms/No. of animals in dose group

Long-Term Studies

The aim of the chronic toxicity studies is the compilation of the profile of a substance after repeated intake of low doses over a longer time period. Three categories can be differentiated: subacute (28 day), subchronic (90 day), as well as chronic (52 weeks and more) in two different mammalian species (e.g., rat/dog). The examination of two clearly different mammalian species reduces false-negative findings caused by potential species specificity, but increases the percentage of false-positive findings. Anyhow, not all relevant effects/ findings can be detected adequately in one species considering sensitivity for man (e.g., teratogenicity of thalidomide). All repeated application studies comprise in general four dose groups (control-, low-, medium-, as well as highest-dose group) whose number can be increased if necessary. The dose range should be spread widely and derived in an algorithmic manner. Ideally, the low dose should show no effects; the medium dose exerts no or slight effects and the highest dose should produce toxicity, but no death or severe suffering. Impaired body weight gain, reduced by at least 10% compared to controls, is typically considered an indication of sufficient toxicity at the top dose.

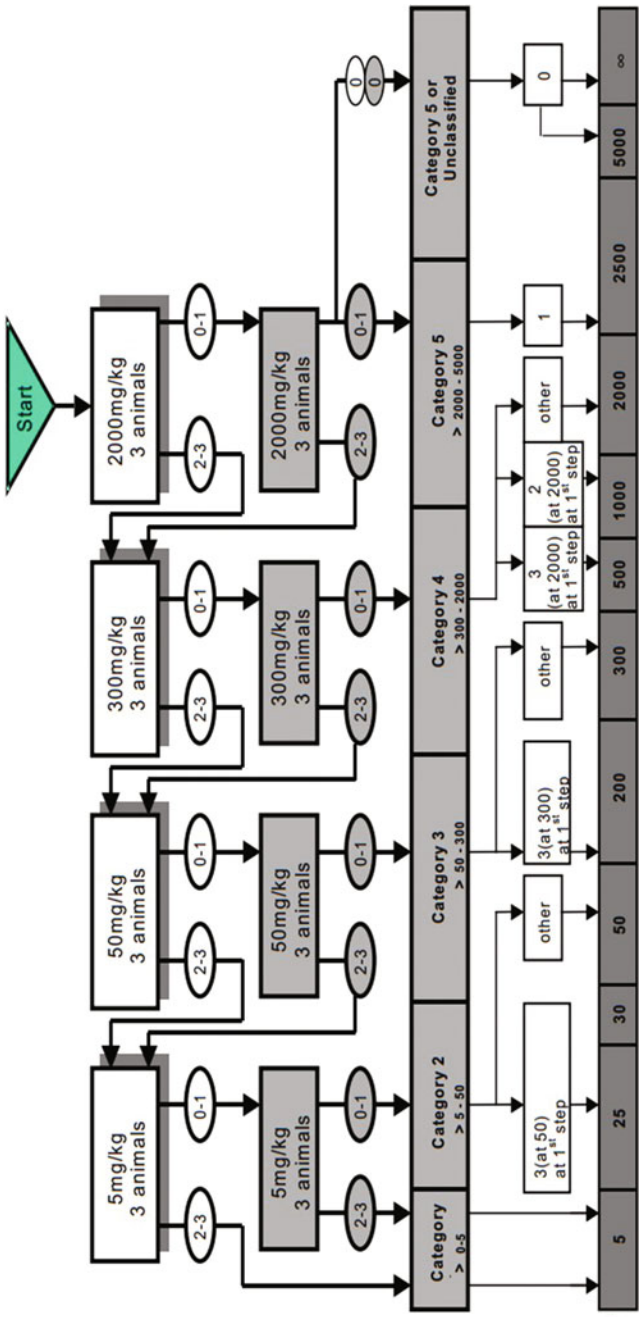

Fig. 2 Acute Toxic Class Method: Rectangles show dose and number of animals used (one sex, preferentially females), figures in ellipses show the number of dead or moribund animals for every step GHS: Globally Harmonized System (mg/kg bw)

Subacute Studies (28 Day)

The subacute study is intended to identify potential target organs and to support dose-range finding for longer-term studies.

Performance Ratio

Besides the investigation of minimum parameters required by the relevant testing guideline, a high-performance ratio should be pursued to reduce the overall number of animal testing for a given substance. Standard investigations need only small amounts of biological material, so that further investigations can be conducted, e.g., into immunotoxicity (thymus, spleen, lymph nodes, Peyer-plaques) or phase-I and -II enzymes in the liver, or a liver foci test as a short-term carcinogenicity test. Such additional investigations can provide valuable information on the toxicological profile and indicate where special attention is needed in the longer-lasting studies (subchronic/chronic) to avoid additional dose groups or even repetition of entire studies.

Dose-Range Finding

A further important function of the subacute study is the dose-range finding for subsequent studies (subchronic/chronic). Based on the large spread of the dose range, one can identify tentative NOELs (no observed effect levels) as well as clear, non-toxic/low toxic doses to determine the profile. In case of doubt, the dose range has to be enlarged.

Reversibility

The reversibility of findings can be studied by adding satellite (recovery) groups. The animals of the satellite group are not treated with the test substance after the application period, whereas all other activities continue over a variable time period in general over 4 weeks. (Non-)recovery of organ functions can be detected as hint to (non-)intact repair mechanisms or adaptation capacities of the organs. Related to the target, the proof of reversibility can be useful also in subacute dermal as well as inhalation studies.

Subchronic and Chronic Studies

Based on the results of the subacute studies, the performance of subchronic/chronic studies is more or less comparable to that of the subacute studies. The basic difference is the treatment period and the number of animals per dose group (subchronic

Table 2 Combined chronic toxicity/carcinogenicity study in rats: schedule of investigations

Check for morbidity or mortality	Twice daily, once daily, on weekend/holiday
Detailed investigations of clinical findings	Once weekly
Functional observation battery	Week 53
Determination of:	
Body weight	Weekly until week 13, then every 2 weeks
Food intake	Weekly until week 13, then every 4 weeks
Water intake	Every 4 weeks
Feeding period	7 days/week
Absolute feeding period[a]	>730 days
Ophthalmological investigations	Start, week 1, 2, 53, 104 (end)
Clinical-chemical investigations:	
Hematology	Week 27, 54, 79, 105
Clinical chemistry	Week 27, 54, 79, 105
nvestigations in urine	Week 27, 54, 79, 105
Investigation of calcium/phosphorus	In bones (optional): 1 and 2 years after start of treatment
Organ weights	1 and 2 year(s) after start of treatment
Interim/end section	1 and 2 year(s) after start of treatment
Anatomic/histopathological investigations	1 and 2 year(s) after start of treatment (including number of tumors and incidences)

[a]Number of days which are used to calculate the food intake

13 weeks, 10–20 animals/dose/sex); chronic from 26 weeks to 52 weeks or 105 weeks as carcinogenicity studies on rats and mice (50–70 animals/dose/sex). For non-rodents (e.g., dog) at least four animals per dose group per sex are used.

The longer treatment period allows detecting effects which need a certain latency period to develop, for example, tumors or secondary effects based on primary impact (idiosyncrasy, late reaction, proliferation of tissue due to permanent irritation). Those effects complete the toxicological profile after repeated application. A typical course (data collection) of a combined chronic toxicity/carcinogenicity study in rats is shown in Table 2.

Definition of NOAELs

Besides the determination of the toxicological profile, chronic studies are used to define a NOAEL (no observed adverse effect level). Considering the definition of NOAELs, there is a prominent influence of the dose range finding on the effect range (profile) as well as the nontoxic range. The term “adverse” is interpreted differently under various regulatory regimes and this interpretation may also change over time. Are transient increases of hepatic phase-I and -II enzyme activities “adverse,” or do they reflect an adaptive physiological reaction of the organ? Which percentage of cholinesterase activity inhibition due to an organophosphate is considered a

toxicological relevant effect and in consequence to be seen as "adverse"? Effects can show a statistical significance without having any biological/toxicological relevance and should therefore not be considered "adverse." In general the NOAELs of subacute, subchronic, and chronic studies serve as point of departure for limit values like ADI (acceptable daily intake), AOEL (acceptable operator exposure level), and others, which are crucial for human health risk assessment. Severe toxic effects pertaining to teratogenicity, genotoxicity, or carcinogenicity can lead to an increase of the safety factor (SF), which accordingly lowers the respective limit value. The treatment period has to be seen in analogy to the expected duration of human exposure, e.g., whether it is incidental (acute) seasonal (subacute/subchronic) or lifelong (chronic).

Cross-References

- ▶ Adverse Effects Versus Non-adverse Effects in Toxicology
- ▶ Aims and Mission of Regulatory Toxicology
- ▶ Dose–Response Relationship and Extrapolation in Toxicology: Mechanistic and Statistical Considerations
- ▶ Examination of Organ Toxicity
- ▶ Toxicity Testing In Vitro: Regulatory Aspects

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Examination of Organ Toxicity

10

Wolfgang Kaufmann and Matt C. Jacobsen

Contents

Abstract

As a result of their anatomy and physiology, the organs and tissues of animals and man may show different morphological responses and sensitivity to xenobiotics. Toxic responses can manifest systemically (e.g., the immune system) or may produce specific toxic effects in a single organ system (skin) or single organ (liver). Organ toxicity may therefore result from a direct and primary effect on a target organ or as a result of secondary effects in organs and tissues that have a physiological dependence on the primary target (Haschek 2009). The

W. Kaufmann
Merck KGaA, Non-Clinical Safety, Global Pathology and Reproduction Toxicology, Darmstadt, Germany
e-mail: Wolfgang.kaufmann@merckgroup.com

M. C. Jacobsen (✉)
Regulatory Safety Centre of Excellence, Clinical Pharmacology and Safety Sciences, IMED Biotech Unit, Biopharmaceuticals R&D, AstraZeneca, Cambridge, UK
e-mail: matt.jacobsen@astrazeneca.com

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assessment of organ toxicity must therefore take into account the complex physiological interdependence of tissues and organs within the body.

Keywords

Toxicologic Pathology · Target Organ · Histopathology · Clinical Chemistry · Organ weight · Necropsy

Introduction

While there have been advances made in the field of in vitro toxicology testing, the safety assessment of novel drugs and agrochemicals relies on in vivo studies utilizing appropriate laboratory animal species. These laboratory animal species are administered the drug or chemical by an appropriate route of administration, and the potential for target organ toxicity is assessed using a variety of endpoints. These endpoints include clinical observations in life, clinical chemistry of blood and urine, necropsy with organ weight measurement, and the collection and histopathology examination of tissues to assess the potential for structural changes associated with toxicity. The robust assessment of target organ toxicity relies on an integration of all these endpoints to provide a weight of evidence approach to assess the potential for toxicity within tissues and organ systems.

Basic Principles for the Examination of Organ Toxicity

Toxicological alterations of the morphology and structure of organs and tissues are detected by anatomical pathology examination following single or multiple doses of a drug or chemical administered to a laboratory species. During the in-life phase and at the end of an animal study, clinical pathology parameters (e.g., hematology and urinalysis) are assessed and provide important biomarkers for functional metabolic disturbances and maybe the first indicators for potential organ toxicity (e.g., liver or kidney). Both anatomical and clinical pathology disciplines offer a broad diagnostic repertoire to analyze organ toxicity in a well-considered, stepwise, so-called tiered approach concept.

Gross Pathology

After the in-life phase of an animal experiment, all study animals are necropsied according to a standardized, systematic procedure. As a rule, the study design is based on the most recent effective guidelines (e.g., OECD guideline for the testing of chemicals or US EPA Health Effects Test Guidelines OPPTS); however, the study design should always be adapted if the mode of action of a chemical is already known. All observations and macroscopic lesions identified at necropsy are recorded in a validated electronic data system for each single study animal. A standard study

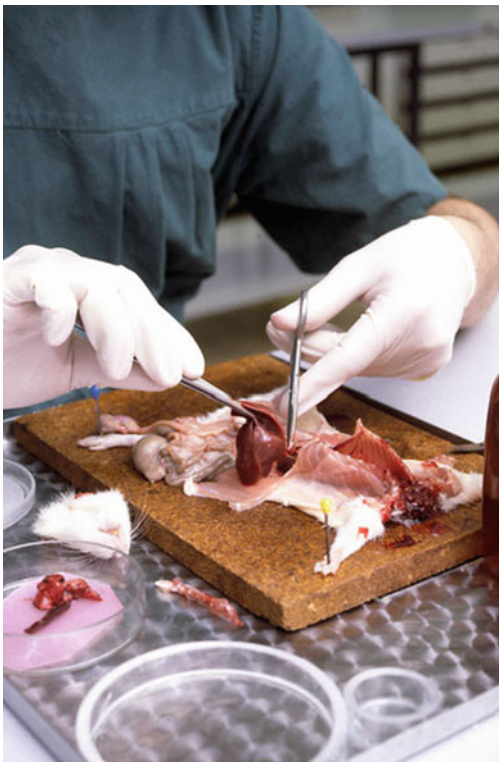


Fig. 1 Necropsy of laboratory animals with organ and tissue collection. The figure shows the removal of the liver from the abdominal cavity of a Wistar rat

protocol lists a wide range of tissues and organs that have to be removed for further histopathology examination. Some selected organs (e.g., liver, kidneys, adrenal glands) are weighed. Organ weights are often sensitive indicators of a treatment-related effect and may provide initial information on potential target organs during or shortly after necropsy (e.g., *increased liver or thyroid gland weights* are often recorded for compounds that act as enzyme inducers). Macroscopic observations during necropsy also have the potential to alert the toxicologist to possible target organs (e.g., *yellow-brown or clay-like discoloration of the liver* is indicative of a degenerative change, and the finding of a *mass* could turn out to be a chronic inflammatory process, an abscess, or a malignant tumor). The careful consideration of organ weights and macroscopic findings are an essential part of the detection of organ toxicity in experimental animal studies (Fig. 1).

Histopathology

The histopathological examination (by light microscopy) of a diverse range of organs and tissues by a well-trained toxicological pathologist is one of the most important and time-consuming elements in the assessment of organ toxicity

(Greaves 2012). With the advances in digital technology, slide scanners can now produce digital whole slide images from histopathology slides that can be reviewed by a pathologist in a similar way to a microscope. The minimum scope for histopathology examination is dictated by the various guidelines already mentioned above. In principle, organs and tissues are selected that are considered highly relevant determinants of basic metabolic and detoxification processes (e.g., the liver), organs that act as portals of entry for drugs/chemicals and may have been in direct contact with the test compound (gastrointestinal tract, lungs, skin), and organs that are crucial for excretion (e.g., kidneys, urinary bladder, and biliary system in the liver). Furthermore, representative samples are also examined from the immune and hematopoietic systems (two lymph nodes, one close to the site of exposure, thymus, spleen, bone marrow), the skeletal system (bone, joints, skeletal muscle), and the nervous system (various coronal sections from the brain including the cerebrum and cerebellum, two to three cross and longitudinal sections from the spinal cord, and one peripheral nerve). The cardiovascular system (arteries, veins) is examined as a constituent of many of the routine organ samples but specifically in one or more targeted sections from the heart and one section of the aorta. The reproductive system (including the testes, epididymides, prostate, accessory sexual glands and ovaries, oviducts, uterus, vagina, and mammary gland) is also included in the organs and tissues examined (Fig. 2).

A thorough histopathological examination is essential as toxicological changes can manifest microscopically in the absence of alterations to organ weights and clinical pathology parameters.

Fig. 2 Histopathology. Scope of examination for one single test animal in a carcinogenicity study (*left* paraffin blocks, *right* histological slides)

Diagnostic Approach, Procedures, and Considerations

As a minimum, all organ and tissue alterations from the high-dose and concurrent control group are recorded systematically in a validated data entry system to produce a pathology finding incidence table. The pathologist chooses an appropriate morphological diagnosis for the lesion observed and may describe the findings in more detail in the narrative pathology report. The pathologist must also grade the severity of findings where appropriate in order to help establish the presence of a dose–response. The grading system used by the pathologist will take into account the type of study (duration of exposure) and the nature of findings observed. The pathologist relies on his knowledge of the spontaneous pathology of the test species used to help differentiate spontaneous from treatment-related findings. A thorough understanding of the potential impact of any histopathology findings on the test species used also helps the pathologist to ascribe adversity to any treatment-related lesions present (Kerlin et al. 2015; Palazzi et al. 2016). The provision of accurate and up-to-date historical control data for spontaneous lesions in test species concerned may also be required to help support the pathologist's conclusions.

There has been an ongoing debate as to whether the study pathologist should have knowledge of which animals are treated and which animals are controls to guarantee a more *objective examination*. However, the approach of *blind reading* is not recommended by toxicological pathologists or their societies for the initial histopathology examination. The risk of introducing "bias" or the potential to overlook or to misinterpret minor treatment-related variations in the morphology of organs is high, and blind reading should therefore not be performed. However, blind reading of histological slides is often used at a later stage during slide evaluation, e.g., to find a no-observed-effect level (NOEL) for a specific organ toxicity and to allow the study pathologist to consistently identify a subtle or borderline lesion. "Blinding" slides with codes can also be performed if there are different opinions on the interpretation of a lesion between pathologists and the lesion is being considered by a pathology working group (PWG). A PWG is a formal and well-documented process to resolve different opinions on the diagnosis and relationship to treatment of pathology findings from a toxicology study by an independent panel of expert pathologists, which also includes the study and peer-reviewing pathologist. These experts undertake a "blind reading" so as not to be biased (Fig. 3).

Clinical Pathology Parameters

In addition to the analysis of anatomical pathology data after completion of an animal study, the analysis of the clinical pathology data will add significant value for the detection of organ toxicity. For clinical pathology, blood and urine samples are taken during the in-life phase of a study at scheduled time points and, as a minimum, at least once before the final sacrifice of the test animals. Diverse hematology parameters are measured and calculated (e.g., number of red and

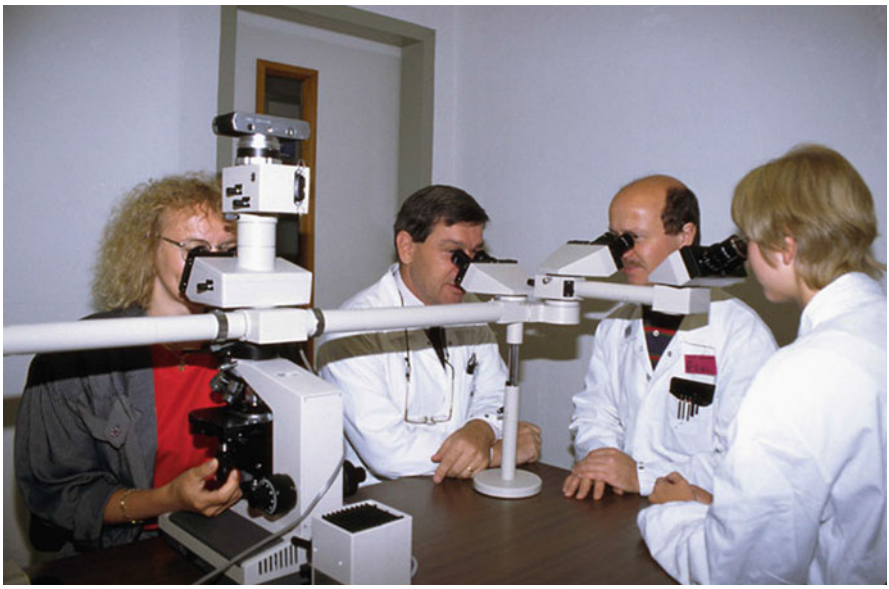


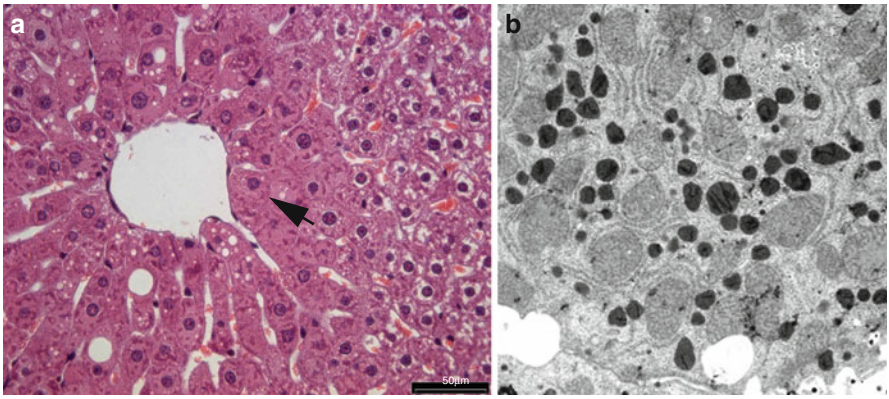
Fig. 3 Evaluation of histological slides by light microscopy. Pathologists during an internal review of histopathological findings at a multiheaded microscope

white blood cells, hemoglobin concentration, mean corpuscular volume, coagulation tests, and differential blood count). As these tests are highly automated, the results can provide an initial assessment of such things as anemia and inflammation prior to the histopathology results. Blood sample analysis also includes clinical chemistry parameters (e.g., enzymes, biochemical analytes in plasma, like transaminases, urea, creatinine, electrolytes, serum protein levels) that may indicate organ toxicity in the liver or the kidneys. As a rule, the selection of the standard minimum panel of clinical pathology parameters in animal experiments follows guideline recommendation and aims to detect major metabolic impacts of a potential toxic compound. If the mode of action of a test item is known, clinical pathology tests may be specifically designed and additional parameters analyzed (e.g., hormones). The clinical pathology data are another important component and, together with the gross pathology, organ weights, and histopathology data analysis, allow the detection of specific organ toxicity with a high degree of certainty.

Results of a Well-Concerted Combination of Anatomical and Clinical Pathology Data Analyses

For the majority of cases, standard approaches like the analysis of hematoxylin and eosin-stained histological slides by light microscopy are sufficient to detect organ toxicity. However, there are also exceptions where more sophisticated methods are required. A liver cell swelling (*centrilobular hypertrophy of hepatocytes*) noted by

light microscope may indicate a degenerative or an adaptive response of the liver parenchyma (Fig. 4a). A degenerative process that leads to liver cell death is much more critical than an adaptive process, where the morphological change is based on a physiological and fully reversible response of the liver tissue. There are a number of different chemicals, so-called enzyme inducers, that produce an adaptive liver cell swelling by a proliferation of the smooth endoplasmic reticulum (SER). A



c

Table
Quantitative measurements of the peroxisomal enzyme activity in B6C3F1 mice

Dose groups	Males	Females
Control	4.03±0.91[a]	5.28±0.41
500 ppm	6.05±1.42**	5.91±0.82
1500 ppm	9.31±2.00***	8.88±0.74***
4000 ppm	23.14±5.33***	22.05±1.60***
8000 ppm	43.37±2.18***	43.90±1.52***

The data show the activity of the cyanide-insensitive palmitoyl-CoA –oxidation. Results are presented as milliunits (mU) per milligram protein as group mean ± standard deviation

*p<0.05
**p<0.02
***p<0.002

Fig. 4 Example of a successful contemporary approach using three different methods to analyze organ-specific lesions and to correlate structure and function: (**a**) Centrilobular liver cell hypertrophy (*arrowhead*) is detected in a histological slide by light microscopy examination. (**b**) The liver cell hypertrophy is characterized by electron microscopy examination as proliferation of specific cell organelles in the cytoplasm, the peroxisomes (here stained as black rounded bodies). (**c**) The clinical pathology examination of the cyanide-insensitive palmitoyl-CoA oxidation in liver homogenates resulted in a statistically significant functional increase and shows a clear dose–response relationship

proliferation of the SER is the morphological correlate for an induction of the cytochrome P450 enzyme superfamily (CYP) and major enzymes in the metabolism of xenobiotics (toxic chemicals and drugs). Morphologically, a definitive diagnosis can be made by using electron microscopy of the liver, and clinical biochemistry allows a diagnosis by the analysis of specific enzymes (e.g., ethoxyresorufin-*O*-deethylase and pentoxyresorufin *O*-dependentylase).

Both methods are also appropriate approaches to identify another group of substances that also induce a centrilobular hypertrophy of hepatocytes, e.g., peroxisome proliferators. The latter result in an accumulation of specific intracytoplasmic cell organelles, the peroxisomes, which play a significant physiological role in lipid metabolism. Peroxisomes can be selectively stained by cytochemical or immunohistochemical methods and can be morphologically quantified. Results from the latter techniques correlate well with a significant increase of the cyanide-insensitive palmitoyl-CoA oxidation that can be analyzed from liver homogenates taken during necropsy (Fig. 4b, c (table)).

These examples show the complementary use of clinical pathology and anatomical pathology approaches to assure the accurate diagnosis and interpretation of certain types of organ toxicity and to contribute to the understanding of the mode of action of xenobiotics.

International Activities on Harmonization in the Use of Diagnostic Terms

For many years, the major scientific societies of toxicological pathology in Europe (European Society of Toxicologic Pathology [ESTP] and British Society of Toxicological Pathology [BSTP]) and the United States (Society of Toxicologic Pathology [STP]) have worked on harmonizing the nomenclature and diagnostic criteria used in toxicology studies. These activities were guided by the need for pathologists globally to use the same pathomorphological diagnostic criteria in the description of findings from toxicity studies using drugs and chemicals. For example, a *hyperplasia of the mammary gland* should be always differentiated from an *adenoma of the mammary gland* using the same diagnostic criteria regardless of geographical location. As pharmaceutical and chemical companies become more globalized, preclinical safety and toxicology studies for a single compound may be performed in research facilities in different geographical regions. As a result of these trends, it was considered extremely important to come to a common understanding in the use of diagnostic criteria. Initially, the primary focus was given to proliferative lesions in rodents as these findings were easier to harmonize among the international community of toxicological pathologists. As a result working groups of toxicological pathologists from Europe and America published a series of *International Classification of Rodent Tumours* for rats and mice between 1992 and 2001. The subsequent use of these published criteria significantly helped to harmonize the diagnosis of tumors in rodent oncogenicity studies. However, there will always be borderline lesions and growth patterns of tumors where harmonized criteria do not fit

the lesion and the pathologist has to make their own informed judgment based on experience.

On the basis of an initiative of the European Society of Toxicologic Pathology (ESTP) and the US Society of Toxicologic Pathology (STP), a further important step forward was started in 2005. In conjunction with the German-based Registry of Industrial Toxicology Animal-data (RITA), a collaborative process of review, update, and harmonization of existing diagnostic nomenclature, documents, and databases of rodents was initiated. The British Society of Toxicological Pathology (BSTP) and the Japanese Society of Toxicologic Pathology (JSTP) joined this process in 2006. This project, known as INHAND (International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice) project, includes efforts to harmonize nomenclature not only for proliferative lesions but also for non-proliferative lesions at a level that gains international acceptance (Mann et al. 2012). Up to 2020, INHAND nomenclature has been published for the cardiovascular (Berridge et al. 2016), integument (Mecklenburg et al. 2013), soft tissues/skeletal (Fossey et al. 2016), respiratory (Renne et al. 2009), hepatobiliary (Thoolen et al. 2010), urinary (Frazier et al. 2012), nervous (Kaufmann et al. 2012), endocrine (Brändli-Baiocco et al. 2018), hematolymphoid (Willard-Mack 2019), special senses (Ramos et al. 2018), reproductive systems (Creasy et al. 2012), gastrointestinal tract (Nolte et al. 2016), and the mammary gland (Rudmann et al. 2012). Guides for non-rodent species are in preparation. The INHAND nomenclature is also available electronically at the goRENI webpage and is presented at the international annual meetings of the societies of toxicological pathology to discuss the practical use of these harmonized diagnostic criteria.

Summary and Future View

Organ toxicity is the result of physiological dysfunction and structural alteration. Clinical pathology and histopathological examination are complementary approaches that underpin the detection and characterization of organ toxicity. Despite the many advances in molecular biology (genomics, metabolomics), the use of routine clinical pathology measurements and histopathological examination of hematoxylin and eosin-stained tissue sections are unlikely to be replaced in the near term as a first-tier approach for detecting organ toxicity in animal toxicology studies. The latter techniques can be complemented by more sophisticated examination using electron microscopy, immunohistochemistry, and molecular pathology approaches, once the target organs have been identified, and may help to identify the mode of action of the toxicity in question.

Steady advances have been made in the validation and use of in vitro systems for predicting organ toxicity. These have mainly involved the evolution of primary cell cultures into more sophisticated microphysiological systems (MPS) that contain more than one cell type (Truskey 2018). With advances in stem cell biology, these MPS can be humanized, to allow for a direct comparison of results between laboratory animals and man.

Cross-References

- ▶ Biomolecules Versus Smaller Chemicals in Toxicology: ICH, EU, and US Recommendations
- ▶ Examination of Acute and Repeated-Dose Toxicity
- ▶ International Regulation of Toxicological Test Procedures
- ▶ Specific Toxicity Tests for Neurotoxicity, Immunotoxicity, Allergy, Irritation, Reprotoxicity, and Carcinogenicity

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11 Specific Toxicity Tests for Neurotoxicity, Immunotoxicity, Allergy, Irritation, Reprotoxicity, and Carcinogenicity

Eckhard von Keutz

Contents

Abstract

The specific test methods used in toxicology classically include tests for reproductive toxicity, genotoxicity, and carcinogenicity. There are now also other test methods such as testing for possible immunotoxic or neurotoxic properties of a substance. Special, usually internationally applicable, test guidelines form the regulatory basis for the test methods, which apply to chemicals, crop protection products, and medicinal products.

Keywords

Reproductive-toxicity · Teratogenicity · Embryo-fetal toxicity · Fertility · Genotoxicity · Point mutation · Chromosomal aberration · DNA damage · Carcinogenicity · 2-year rat study · Neurotoxicity · Immunotoxicity

E. von Keutz (✉)
Pharmaceuticals, Translational Sciences, Bayer AG, Wuppertal, Germany

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Introduction

In principle, toxicity testing can be separated into two major groups. One is more general in nature and includes (general) toxicity tests ranging from acute (single-exposure) studies to repeat-dose (multiple-exposure) studies, whereas the other group involves (specific) toxicity tests which examine specific types of adverse effects, also known as endpoints. Such endpoints cover well-known risks such as reproductive toxicity, genotoxicity, and carcinogenicity but also very specific risks such as immunotoxicity or neurotoxicity. The aim of this chapter is to describe the current regulation and the specific test methods used in toxicology to elucidate and characterize these particular risks. Most of the test guidelines (TGs) mentioned in this chapter are based on the internationally harmonized TGs for chemicals (see OECD 2020) and the harmonized TGs for pharmaceuticals (see ICH 2020).

In the past, most toxicity tests were performed in animals, and as a consequence, today's regulation is still mainly based on results obtained in animals. It should, however, be mentioned that in line with the protection of animals used for scientific purposes in the meantime, basically all legislations that rely on toxicological information either require or strongly encourage the use of alternatives to animal testing. With new technologies and alternatives becoming available (for more details, see ► Chap. 12, "Toxicity Testing In Vitro: Regulatory Aspects"), it can be expected that new testing strategies and testing methods will be evaluated also in the context of specific toxicity tests and will find their way into new regulations (EMA 2020; EPA 2020; FDA 2020).

Reproductive Toxicity Testing

The importance of reproduction toxicology as part of the assessment of safety gained sad notoriety in the wake of the thalidomide (Contergan) tragedy. At that time, routine testing for possible teratogenic properties of a substance was not yet established. This was because such a possibility was not expected on the basis of the scientific knowledge back then. We now know that chemical substances are fundamentally capable of causing damage in all stages of reproduction. The maturation of gametes can be disturbed in women or men, for example. But the release of mature gametes can also be disturbed, as can fertilization; cell division; egg implantation in the uterus; intrauterine development, i.e., the development of organs during the embryonic phase and fetal maturation; and development after birth (postnatal phase). In order to clarify whether and at what point in time reproductive effects can occur, the treatments must be carried out during specific periods of time. A distinction is therefore made between testing for effects on male or female fertility (from spermatogenesis/follicle maturation to implantation), on intrauterine development (during organogenesis), and on peri- and postnatal development (from fetal development to the end of lactation).

Testing for possible *teratogenic/embryotoxic properties* is now mandatory for all substance classes. The procedures for carrying out this testing are described in

publications including the *OECD Guideline for Testing of Chemicals* No. 414 ("Teratogenicity") and in ICH Guideline S5(R3) ("Guideline on reproductive toxicology: Detection of toxicity to reproduction for human pharmaceuticals"). Testing is based on the principle that two different animal species (usually the rat and the rabbit) are exposed to the substance to be tested during the most sensitive stage of pregnancy. Immediately before the natural end of pregnancy, the fetuses are delivered by cesarean section and then examined for external and internal malformations. Rats are treated from day 6–15 and rabbits from day 6–18 of pregnancy. The most important organs are formed during these periods. Any teratogenic/embryotoxic potential a substance may have should therefore have its greatest impact under this treatment regimen and should therefore also be detectable with the greatest degree of reliability. While in the past the majority of data was generated in animals, current testing strategies are expected to consider minimizing the use of animals. Alternative assays and/or in vivo studies with fewer animals can be used to identify hazards in a tiered manner. The use of qualified alternative assays is appropriate for risk assessment under certain circumstances where they are interpreted in conjunction with in vivo reproductive testing. Although they are not a replacement for all in vivo reproductive testing, they can reduce in vivo animal studies and/or animal usage (see also EMA 2020).

One- or two-generation studies in rats, conducted for chemicals and crop protection products, represent additional test methods that investigate the effects of a substance on male or female fertility and on progeny. The methods are described in the *OECD Guidelines for Testing of Chemicals* No. 415 ("One-Generation Reproduction Toxicity Study") and 416 ("Two-Generation Reproduction Toxicity Study"). For medicinal products, the legislation stipulates testing for *effects on male or female fertility* in the rat (ICH Guideline S5 (R3)) and *testing for pre- and postnatal toxicity*, also in the rat (ICH Guideline S5 (R3)), in addition to testing for teratogenic or embryotoxic properties.

Study objectives and common study types:

- Testing for embryotoxic or teratogenic properties in the rat and rabbit (embryo-fetal developmental (EFD) toxicity study)*
- Testing for effects on male or female fertility in the rat*
- Testing for pre- and postnatal toxicity in the rat*
- One- or two-generation studies in the rat

*Some of the endpoints may be combined in one study, e.g., the combined fertility and embryonic development study (FEFD) or the combined fertility and pre- and postnatal development study (FPPND).

There are also guidelines available which classify reproductive risks. For chemicals, there is guidance on classification of reproductive toxicity under the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). This document offers guidance on the determination of level of concern as a measure of adversity and the level of evidence to ascribe classification based on data from tests in laboratory animals. For pharmaceuticals, there is a guideline on risk

assessment of medicinal products on human reproduction and lactation. This document describes how to assess the risk of adverse reproductive/development effect in humans based on reproductive toxicity studies in animals and human clinical data. It addresses information to be included in the summary of product characteristics on how to use the medicinal product taking into account the nature of the risk.

Genotoxicity Testing

The strategy for testing for genotoxicity covers three main areas, namely, gene mutation, chromosome aberration or breakage (clastogenicity), and chromosomal loss or gain (aneuploidy). Testing for genotoxic properties (effects on the cell's genetic material) is mandatory for most substances. A multitude of test methods (in vitro and in vivo) are available for this, and these can be used to detect a variety of harmful effects. More specifically, these include gene or point mutations, structural chromosome changes (chromosomal aberrations), and changes in chromosome number (changes in the DNA). Mutations are significant for humans in a number of respects. On the one hand, mutations in somatic cells can pave the way for cancer, and they therefore have a direct effect on the individual concerned. Germline mutations, on the other hand, can lead to prenatal death or to malformations ("hereditary diseases") in offspring. They therefore have effects on subsequent generations.

Because ***point mutations*** are not visible under the microscope, the induction of these mutations is detected indirectly via their effects. These consist mainly of protein changes, with enzyme functions often being used as a means of detection. Bacteria are eminently suited to this task, as they enable individual mutants to be detected among millions of cells with the aid of selection media. Probably the best-known mutagenicity test of all is the Ames test, which will be described briefly here as an example of a method for detecting point mutations. The object of the test is a defective (mutant) strain of *Salmonella typhimurium* that is no longer capable of synthesizing histidine. The mutated bacteria have the ability to revert to the normal wild type under the influence of mutagens. A concentration-dependent increase to the point of histidine independence is considered as evidence of point mutagenicity. Because of the simplicity of the method, the Ames test is a suitable screening test for many substances. It is a quick and sensitive assay. A high degree of mathematical correlation has been shown in some cases by comparison of the mutagenic effects of substances in the Ames test and their carcinogenic effects in animal studies.

In vitro tests to detect ***chromosome or ploidy mutations***, known as "cytogenetic studies," can generally be performed with all primary or permanent cell lines possessing a relatively constant set of chromosomes. Chinese hamster, mouse, and rat cells are used most often, although human lymphocytes are also used. In vivo studies are usually conducted in small mammals, and the assay in Chinese hamster bone marrow can be mentioned as an example of the basic procedure. The test substance is administered to the animal which is then sacrificed after exposure of the organism to the substance for a period of time. Shortly before sacrifice, cell division

is arrested at metaphase by administration of a spindle inhibitor. The bone marrow is removed from the sacrificed animal and examined under the microscope for chromosomal changes.

Another option for testing consists of investigating whether a substance has caused *DNA damage*. Historically, the preferred assay for measuring DNA damage has been the rodent liver unscheduled DNA synthesis (UDS) assay, but this has been superseded in the meantime by the single cell gel electrophoresis (SGCE) assay or comet assay. The comet assay is a versatile, reliable, cost-efficient, and fast technique for detecting DNA damage and repair in almost any cell type and applicable to both eukaryotic and prokaryotic organisms.

Detailed descriptions of the test methods can be found in the OECD Guidelines (*OECD Guidelines for Testing of Chemicals* Nos. 471–486). For medicinal products, the corresponding information can be found in ICH Guideline S2 (R1).

Study objectives and common study types:

- Testing for point mutations (e.g., Ames test in *Salmonella typhimurium* strains)
- Testing for chromosomal aberrations
- In vitro, e.g., cytogenetic studies in Chinese hamster cells
- In vivo, e.g., tests using hamster bone marrow or micronucleus test in the mouse
- Testing for DNA damage (e.g., comet assay in vitro and in vivo)

It is not possible to assess the possible mutagenic risk of a substance on the basis of just one test. Instead, the different ways in which damage can be caused must be addressed and studied on the basis of specific endpoints. This can only be done within the context of a testing strategy in which the test systems must be considered hierarchically. A combination of a bacterial test (e.g., Ames test using various strains of *Salmonella typhimurium*), an in vitro test in mammalian cells (e.g., cytogenetic studies in Chinese hamster ovary cells), and an in vivo study (e.g., micronucleus test in the mouse) represents a standard battery of tests. If a substance has been tested sufficiently for mutagenic properties without any evidence of mutagenic potential being found, it can be assumed that the risk to humans is negligibly small. The risk assessment in the presence of positive findings in lower organisms which cannot be confirmed using relevant methods in mammalian organisms is more complex. As a general rule, for the assessment of any potential risk a substance might pose to humans, the significance of the method increases the more the test system corresponds to the conditions in mammals. If in vivo studies in mammals yield positive findings, this must be seen as a clear indication of the possibility of mutagenic effects in humans. Because positive findings must also always be viewed in the context of a risk of cancer, they are of predictive significance in the assessment of the possible carcinogenic potential of a substance. Herein may lie the true value of these tests that are so quick and easy to perform. Positive findings in genotoxicity studies must always be taken seriously and require careful further investigation. The question of what risk must be deduced from the results of genotoxicity studies can be answered only after an assessment of all the studies. The ultimate classification and evaluation of the findings must take place within the overall context of the risk/benefit assessment.

Carcinogenicity Testing

One of the most complex toxicological tests is the testing of a substance for possible tumorigenic (carcinogenic) properties. The tests are usually performed in two rodent species, specifically rats and mice, and more rarely in hamsters. Ideally, the substance to be tested should be metabolized similarly in the animal species used to the way it is metabolized in humans. The study duration is generally 24 months in rats and 21–24 months in mice and hamsters, depending on the animal strain used, and thus covers the majority of the life expectancy of the study animals. This mimics almost in fast motion the lifelong exposure to a substance in humans. The way the substance to be tested is administered is guided by the conditions in humans (administration in the feed, in the drinking water, by gavage, by inhalation, etc.). The testing includes three-dose groups and an untreated control group. Fifty animals of each sex are generally used per dose level. The doses are selected in such a way that there are clear intervals between them. The highest dose should be close to the maximum tolerated dose (MTD). If this dose were exceeded, the animals would die from the effects of the substance before it was possible for cancer to develop in the first place. Administering the maximum tolerated dose makes the carcinogenicity study particularly sensitive. This is also necessary because it is only ever possible to study the substance in a limited number of animals, although the risk needs to be assessed for a large number of exposed people. The exact procedure for conducting carcinogenicity studies is described in the OECD Guidelines (*OECD Guidelines for Testing of Chemicals* Nos. 451/453).

A few particularities need to be taken into account in the testing of medicinal products for possible tumorigenic properties. The option exists, for example, to replace the long-term study in mice with a meaningful short-term test. Various transgenic animal models can be used for this. Which model is the most suitable must be decided depending on the substance and the particular parameter(s) being studied. Ideally, this should be done in close consultation with the competent authorities. In the case of medicinal products, the dose is selected taking into account pharmaceutical considerations, with a key role being played by the comparison of human/animal exposure on the basis of the achieved/achievable plasma concentrations. Information on conducting carcinogenicity studies for medicinal products can be found in ICH Guidelines S1A, S1B, and S1C (R2). It should be mentioned that for medicinal products, analyses are ongoing in order to explore new and eventually better ways to predict a carcinogenic potential. The results of these analyses hold promise in driving to support modifications to current carcinogenicity testing guidelines while maintaining patient safety, accelerating patient access, and significantly reducing animal testing.

Basic structure of carcinogenicity studies:

- Two rodent species (rat and mouse or hamster)
- Three-dose groups and an untreated control group
- High numbers of animals (50 animals of each sex per dose level)
- Lifelong treatment (24 months)

- Highest possible dosages (maximum tolerated dose = MTD or exposure calculations, if relevant)

The crucial evidence in respect of the outcome of carcinogenicity studies is provided by the necropsy of the study animals and the subsequent histopathological examination. There are no hard and fast rules on how the results of carcinogenicity studies, whether positive or negative, should be evaluated. An assessment of the risk can be performed only on the basis of well-planned and conducted studies and by a committee of experienced top specialists.

As mentioned above, for medicinal products, efforts are ongoing to introduce a more comprehensive and integrated approach to address the risk of human carcinogenicity and to define conditions under which the 2-year rat carcinogenicity studies add value to that assessment. There is growing evidence that knowledge of pharmacological targets and pathways together with toxicological data can, in certain cases, provide sufficient information to anticipate the outcome of the 2-year rat carcinogenicity studies and their predictive value for a given pharmaceutical. Based on this knowledge, it may be possible to conclude that a given pharmaceutical in certain cases presents a negligible risk or, conversely, a likely risk of human carcinogenicity without conducting a 2-year carcinogenicity study. This hypothesis is currently tested by a prospective evaluation.

Neurotoxicity Testing

Specific tests for neurotoxic effects are required for crop protection products in particular. The range of tests covers three main elements: the "functional observational battery" (FOB), motor activity (MA), and neuropathology.

In the *functional observational battery (FOB)*, a series of noninvasive tests is performed which can be used to detect and quantify behavioral abnormalities and neurological effects in the study animals (usually rats). The initial focus is on close observation. Even the tiniest changes in posture, appearance, and movement are noted, with a distinction being made, for example, between observation of the animal in its own cage, during handling, and outside the cage (on a free surface). In addition, a range of responses (including the approach, touch, noise, and tail-pinch responses) and reflexes (including pupil response, righting reflex, and grip strength) are assessed. The basic requirement for the reproducibility of these tests is the standardization of the test conditions. This includes, for example, all the animals in a study always being assessed by the same investigator. For *motor activity (MA)* testing, the animals are placed in chambers equipped with infrared light barriers and their movements observed closely. The automated recording of findings enables even the tiniest changes in the motor activity of the study animals to be recorded. Possible substance effects can result in an increase or decrease in motor activity. Testing for neurotoxic effects includes a thorough *neuropathological examination*.For this purpose, different localizations of the central and peripheral nervous system are prepared at the end of the study using specific techniques

and examined and assessed in respect of morphological effects. A detailed description of the test methods can be found in publications including the *OECD Guideline for Testing of Chemicals* No. 424 and the EPA Health Effects Test Guidelines (OPPTS870.6200).

Key elements of neurotoxicity testing:

- Functional observational battery (FOB)
- Motor activity (MA)
- Neuropathology

Possible neurotoxic properties of a substance can have particularly serious consequences for the developing organism. It is therefore not surprising that particular importance is attached to this aspect in connection with specific tests, the main focus of interest being the recording of behavioral changes and neurological deficits in progeny/young animals.

Immunotoxicity Testing

Testing substances for possible immunotoxic properties has taken on much greater prominence in recent years and is now established in numerous test guidelines.

According to the FDA (1999), immunotoxicity refers to any adverse effect on the structure or function of the immune system, or on other systems as a result of immune system dysfunction. An effect is considered adverse or immunotoxic if it impairs humoral or cellular immunity needed by the host to defend itself against infectious or neoplastic disease (immunosuppression) or it causes unnecessary tissue damage (autoimmunity, hypersensitivity, or chronic inflammation). This definition incorporates the concept that the immune system is in a complex balance with other systems (e.g., nervous and endocrine) that may utilize or be affected by the same biological mediators (e.g., neuropeptide and steroid hormones).

Some immunotoxicological tests have been an established component of toxicological testing for many years. This applies to investigations of the possible skin-sensitizing/allergic potential of a substance, for example. The *Buehler test* and the *Magnusson and Kligman maximization test* represent typical methods for detecting these kinds of reactions. Both tests are conducted in guinea pigs, with a distinction being made between adjuvant (maximization) and non-adjuvant (Buehler) tests. Freund's adjuvant is administered additionally to boost any immune response induced by the test substance in the test concerned. The principle of the test consists of the animals, after initial exposure to the substance ("induction") and after a waiting period (generally 14 days) has elapsed, being confronted with the substance a second time ("challenge"). The responses which then occur are used to assess whether the substance has skin-sensitizing potential or not. A detailed description of these tests can be found in the *OECD Guideline for Testing of Chemicals* No. 406 and in the EPA Health Effects Test Guidelines (OPPTS870.2600). A more recent test is represented by what is known as the *local lymph node assay*, which is described in

the *OECD Guideline for Testing of Chemicals* No. 429. For medicinal products, the aspect of skin sensitization plays a role with dermal dosage forms in particular. Testing is required specifically in the European "Note for Guidance on Non-Clinical Local Tolerance Testing of Medicinal Products" (CPMP/SWP/2145/00).

Some of the parameters studied in standard toxicological testing can themselves provide information on immune system involvement. These include hematological parameters (white blood cell count, differential blood count), clinical chemistry parameters (protein electrophoresis and albumin/globulin ratio), organ weights (spleen, thymus, lymph nodes), and especially histopathological examination of the spleen, lymph nodes, Peyer's patches, thymus, and bone marrow. Specific tests are now also available in addition to these standard parameters. These are functional tests such as the *plaque assay*, which involves immunizing the test animals against sheep red blood cells a few days before necropsy and measuring the resulting immune response on the day of necropsy, or more in-depth cytofluorometric analyses of *lymphocyte subpopulations* in the spleen and blood using FACS. If any of these tests yield evidence of immunotoxicity, the range of tests must be expanded ("tier approach"). In such cases, consideration should be given to performing a *host resistance (HR) assay*, for example, in which the treated animals are infected with bacteria or viruses, and any impairment of immunity by the substance is measured.

The exact procedure for testing for immunotoxic properties of a substance is described in various test guidelines. Specific reference should be made here to the EPA Health Effects Guideline, "Immunotoxicity" (OPPTS 870.7800) and the ICH Guideline S8 ("Immunotoxicity Studies for Human Pharmaceuticals").

Cross-References

- ▶ Aims and Mission of Regulatory Toxicology
- ▶ Defence of Biocidal Active Substance Dossiers in the Context of Regulatory Toxicology
- ▶ Examination of Acute and Repeated-Dose Toxicity
- ▶ Integration of Advanced Technologies into Regulatory Toxicology
- ▶ Omics in Toxicology
- ▶ Toxicity Testing In Vitro: Regulatory Aspects

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Toxicity Testing In Vitro: Regulatory Aspects

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Eckhard von Keutz

Contents

Abstract

In vitro testing in toxicology was limited for a long time to testing for possible genotoxic properties of a substance. Cell or tissue culture methods are now used for the early toxicological assessment of new substances within the context of screening tests and for mechanistic investigations. In this respect, in vitro methods represent a valuable adjunct to animal studies, without being able to replace animal studies completely at the present time. New developments in the area of microphysiological systems (MPS) suggest that in specific cases like the development of biologics as pharmaceuticals, where relevant animal models are not available due to the human-specific nature of the biologic, but also for improved mechanistic evaluations, human in vitro models will be required or at least deliver helpful information.

E. von Keutz (✉)
Pharmaceuticals, Translational Sciences, Bayer AG, Wuppertal, Germany

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Cell cultures · Cell lines · Screening · Microphysiological systems · 3D spheroids · 3D bioprinting · Organ-on-a-chip · Stem cells · Ion channels · Phototoxicity · Cardiotoxicity · Genotoxicity

Introduction

"In vitro" tests are known colloquially as "test tube experiments," i.e., they are performed outside the living organism. According to this definition, in vitro testing encompasses tests with isolated organs or tissues, cells, cell organelles, receptors, or ion channels. The culture of cells from different organs is the in vitro method most commonly used in toxicology, with a distinction being made between *primary cell cultures* and permanent *cell lines*. Primary cell cultures are prepared from freshly isolated cells obtained during a necropsy or from surgical specimens, for example. Permanent cell lines, such as tumor cell lines, are obtained from cells which have been transformed spontaneously or in a targeted manner such that they can be passaged without limit and kept in stock. They can now be acquired easily from cell banks. Another source for human cells is stem cells. *Adult stem cells* are isolated from donor fetal or adult tissue(s), whereas *pluripotent stem cells* can be isolated from the inner cell mass of the blastocyst, as with *embryonic stem (ES)* cells, or through nuclear reprogramming, as with induced pluripotent stem cells (iPSC). Which culture systems should be used must be decided individually, depending on the specific parameter(s) being studied.

With regard to their aims, in vitro tests can be viewed in two ways: firstly, as a method of screening and, secondly, in the context of *mechanistic evaluation*. Whereas pharmacological research has been making use of cell and tissue cultures for a long time in efficacy testing of new medicinal products, the use of such systems in toxicology was previously limited to just a few areas of investigation. In vitro methods now represent a vital tool in the early toxicological characterization of new substances, however. This can be attributed, using pharmaceutical research as an example, to the significant increase in efficiency (higher output) and the resulting need to subject potential candidates for development to toxicological assessment at an early stage and to support optimization. It is clear that, because of their low throughput and the large amounts of substance required, conventional toxicological methods, and animal studies in particular, are not able to meet the requirements placed on screening. In vitro methods, on the other hand, have low substance requirements; they can be performed quickly and they are in many cases cheaper. The relatively simple in vitro systems do, however, often show limitations when it comes to generating data on a substance about which little or no previous information exists. This kind of information can usually be provided only by methods with a high level of complexity, which naturally cannot be achieved with simple in vitro methods used for screening. The particular value of in vitro methods therefore lies in the investigation of questions arising on the basis of specific evidence from an

animal study. In this connection, early screening must always be seen as *screening via knowledge*, i.e., based on previous information. Once a data pool generated under in vivo conditions is available, this can be examined in detail with the aid of cell or tissue culture methods. Another particular benefit of in vitro methods lies in the fact that human material (such as surgical specimens) can be used, and the basis for human risk assessment can thus be improved. In vitro methods can therefore represent a useful adjunct to animal studies with the possibility of evaluation of the human relevance of findings obtained in animals.

Possible uses of established in vitro screening models are the following:

- Detection of cellular toxicity in defined organ models
- Testing for specific toxicity (e.g., phototoxicity)
- Tests using receptors or ion channels in the context of safety pharmacology studies
- Genotoxicity testing

Some of the currently common in vitro methods and their possible uses are described below. New developments in the area of MPS which strive for in vivo-like complexity will be described in a separate chapter.

Tests with Liver Cell Cultures (Biotransformation and Cytotoxicity)

The liver plays a central role in the metabolism of foreign substances. Its extraordinary capacity to convert and break down substances is largely attributable to the parenchymal cells of the liver or hepatocytes. From a toxicological point of view, the liver is one of the most important target organs for toxicity. It is therefore not surprising that, in the context of in vitro testing, particularly great importance is attached to tests using isolated liver cells. Hepatocytes from the common study animal species (mouse, rat, dog, or monkey) are relatively easy to obtain because most toxicological studies end with necropsy of the animals. Obtaining human tissue is more difficult, and use must be made here of surgical specimens obtained, for example, from resected liver tissue, following tumor surgery. The hepatocytes obtained at necropsy or from surgical specimens via perfusion are used as primary cultures. They can be maintained in culture for days to a few weeks depending on the culture method, but lose their full functionality over time, with a particularly sharp decline in their cytochrome P450 enzyme (CYP) activity. The use of newer culture methods such as sandwich culture, in which primary hepatocytes are sandwiched between layers of a collagen matrix or cultured as 3D spheroids, potentially as a coculture with non-parenchymatous liver cells, significantly prolongs the period for which metabolic activity can be maintained. Under these conditions, it is possible to maintain hepatocytes in culture for up to 14 days with rather stable basal and inducible CYP metabolic activity, although at a likely lower level than in vivo (Gómez-Lechón et al. 2014).

Tests on the metabolism and cytotoxicity of substances can be performed with liver cells obtained and maintained in culture in this way. Preliminary statements can thus be made about their biotransformation without animal studies or trials in humans having been conducted. Within the context of drug development, these tests are therefore also of particular importance because the results obtained in human hepatocytes can be compared with the results from the hepatocytes of the study animal species in which the toxicological studies have been or should be carried out. On the basis of the comparability of the metabolic pattern (human compared with animal in vitro), conclusions can then be drawn as to whether the study animal species used in the toxicological studies can be classified as relevant in terms of their applicability to humans.

Cytotoxicity tests with liver cells involve measuring the levels of certain enzymes in the culture supernatant. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) can be mentioned here as examples. Increased levels of these (cellular) enzymes in the supernatant are an indicator of cell damage, following the same principle as is applied in the diagnosis of liver damage in patients (increased levels of liver-specific enzymes in the blood as an indicator of liver damage). Determination of mitochondrial dehydrogenase activity (e.g., with the MTT assay) allows to assess the effect of a compound on the metabolic activity of hepatocytes, which essentially correlates with the level of viable cells in an incubation. The use of liver cell cultures in the context of early screening has proven particularly effective when previous information is available from in vivo studies. Under these conditions ("screening via knowledge"), tests with hepatocytes can be used for further chemical optimization with a view to avoiding or eliminating hepatotoxic properties in a new substance. Inconclusive findings from animal studies or significant species differences resulting in uncertainty in assessing the possible risk to humans provide the basis for another possible use. In these cases, testing with human specimens can improve the basis for the risk assessment. Although tests with liver cell cultures are not a regulatory requirement, they represent an important internal decision-making criterion in the context of substance preselection or supplementing/supporting in vivo data with regard to their applicability to humans.

Tests with Mouse Fibroblasts (Phototoxicity)

The term "phototoxicity" is used to refer to reactions triggered when an organism exposed to light or the sun shows particular sensitivity to certain (phototoxic) substances, resulting in harmful health effects. These kinds of reactions can range from local symptoms resembling sunburn (redness) to severe burns (extensive skin necrosis) and general health effects. A large number of substances (especially cosmetics, medicinal products) are now known to have phototoxic potential. It is therefore appropriate that the legislation requires manufacturers to provide information on phototoxicity in the presence of relevant grounds for suspicion (photo instability, presence/accumulation of the substance in the skin) or for certain indications (dermally applied substances). Traditionally, these kinds of tests were

performed in animals and involved mice, rats, guinea pigs, or rabbits being irradiated with UV light after being treated with the substances to be tested.

The most widely used in vitro assay for phototoxicity is the "in vitro 3T3 Neutral Red Uptake Phototoxicity Test" (3T3 NRU-PT) for which a guideline (OECD 2004) is available, which has recently been updated (OECD 2019). The assay was developed under the leadership of the Center for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET) of the Federal Institute for Consumer Health Protection and Veterinary Medicine (BgVV). It is performed using a permanent mouse fibroblast cell line (Balb/c 3T3) and is based on the testing and comparison of the cytotoxic effects of a substance in the presence or absence of exposure to UV light. This in vitro assay is also part of a sequential phototoxicity testing strategy proposed in a CPMP guidance document (CPMP/SWP/398/01 2002), and it is also mentioned in an ICH guideline for the testing of pharmaceutical drugs (CHMP/ICH/752211/2012 2015). While it is acknowledged that the 3T3 NRU-PT assay is a very sensitive test and many positive findings are not confirmed in in vivo follow-up studies, the importance of the 3T3 NRU-PT assay within the context of a sequential testing strategy lies in the fact that if a negative result is obtained, i.e., if evidence is obtained of the absence of phototoxicity, no other tests, and specifically no animal studies, need to be performed, something which is to be greatly welcomed from the point of view of limiting the number of animal studies. However, a positive result in the 3T3 NRU-PT should not be regarded as indicative of a likely clinical phototoxic risk, since it is primarily a hazard identification test. Even in the case of a positive in vitro phototoxicity test, the concentration at which cytotoxicity is observed in vitro after UV irradiation can be used for calculation of safety margin in the context of expected human blood concentrations at therapeutic dose levels.

Tests with Isolated Ion Channels (Cardiotoxicity, ECG Changes)

In the context of the risk assessment of medicinal products, possible cardiotoxic properties, particularly in medicinal products used primarily in non-cardiovascular indications, have been the focus of attention for some time now. The properties concerned are characteristic ECG changes (prolongation of the QT interval as evidence of delayed cardiac repolarization) which are considered predictive in respect of the induction of arrhythmias. This kind of potential must be identified at an early, i.e., preclinical, stage, and the legislation therefore consistently requires appropriate nonclinical (safety pharmacology) studies. Reference should be made in this connection to an ICH test guideline entitled "The non-clinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals" (CPMP/ICH/423/02 2005). Among the tests stipulated in this test guideline are tests using human potassium channels which are expressed in a cell line in a stable manner (hERG). The background for the tests is the fact that cardiac repolarization is essentially mediated by potassium flow and that drug-induced inhibition of the ion channels leads to prolongation of the action potential, which

makes itself apparent in the ECG in the form of prolongation of the QT interval. Tests using hERG channels represent an additional and new example of in vitro studies that are established in regulatory terms. Enhanced evaluation of pro-arrhythmic risk is now being conducted by international initiatives including the Comprehensive in vitro Proarrhythmia Assay (CiPA) and the Japanese iPS cardiac safety assessment initiatives (Kanda et al. 2018; Blinova et al. 2018). The CiPA approach is based on several key modalities including (1) testing of further major ion channels in addition to hERG in correspondingly transfected cell lines, (2) in silico modeling of the ion channel effects, (3) proarrhythmic assessment in human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), and (4) clinical assessment of electrocardiograms from phase I human studies.

Most recent developments in in vitro cardiotoxicity testing expand the use of hiPSC-CM, by testing not only for proarrhythmic risk but also for other relevant parameters like compound-induced effects on changes in contractility, voltage, and/or Ca handling of cardiomyocytes (Saleem et al. 2020).

Tests with Mammalian Cell Cultures (Genotoxicity)

In vitro methods are already long established and stipulated by test guidelines as standard in the field of genotoxicity testing. These kinds of test were in fact already in use even before the development and use of in vivo methods. For the testing of a substance for genotoxic properties, it is assumed that no single test system is capable of predicting a possible risk to humans in a reliable manner. This is why batteries of tests are used to test substances for possible genotoxic effects. A typical battery of tests, stipulated for the testing of medicinal products, for example, consists of two in vitro tests (gene mutation test in bacteria, chromosomal aberration test in mammalian cell cultures) and one in vivo test (micronucleus test in the bone marrow) (Galloway 2017). To perform chromosomal aberration tests under in vitro conditions, cells in culture are treated with the substance to be tested in both the presence and absence of external metabolic activation, arrested at metaphase by administration of a spindle inhibitor, fixed, and then evaluated under the microscope. Permanent fibroblast cells originating from various Chinese hamster tissues are most often used for these tests, including V79, CHO, or CHL cells. These are particularly suitable for chromosome analyses because of their small number of chromosomes and especially their uncomplicated karyotype. Human peripheral lymphocytes are also used.

Tests with Incubated Chicken Eggs (Various Parameters)

Incubated chicken eggs have long been a well-established test system in biomedical research. Development stages without any sensitivity to pain are also used as a model. Both the embryo and the extraembryonic vascular systems are considered as target structures. The substances to be tested are administered directly or via the

intravascular route. Functional and/or morphological parameters are end points. The results of numerous studies show that incubated chicken eggs are used to determine the irritation potential; to screen for cardiovascular effects, phototoxicity, and angiogenesis; and in cancer research. More recently and since in vitro cell culture tests have been developed for these toxicities, testing with incubated chicken eggs is being used less frequently, especially for skin and eye irritation.

Human Embryonic Stem (hES) Cells for Use in Toxicity Testing, E.g., Early Development Toxicity Testing

Early developmental toxicity assays for screening of various compounds for the potential risks for abnormal development in the growing embryo have been traditionally based on animal cells. As species differences might affect the accuracy of the assessments, there is an increasing need for alternative cell sources. In vitro differentiation of hES cells bears a resemblance to the early stages of human embryonic development and offers in principle the possibilities for alternative toxicity testing.

Developmental toxicity evaluations, as used in safety assessment assays, are sometimes suffering from a lack of normal, reproducible, and easily available human cell systems. In this context, pluripotent hES cells and their derivatives have the potential to improve the quality of targets, hits, and leads, thus reducing late-stage attrition. The promise of hES cells for in vitro toxicology is the indefinite access to starting material of identical origin in combination with highly human-relevant assays for, e.g., developmental toxicity testing (Luz and Tokar 2018). Very recently such developmental toxicity testing in stem cells has been evaluated in hiPSC, which yielded promising results with a list of relevant reference compounds (Aikawa 2020).

Possibilities and Limitations of Established In Vitro Toxicity Testing

It is no longer possible to imagine modern toxicity testing without in vitro test systems. They enable initial information on toxicological properties to be obtained within the context of early screening and can provide chemical research with important indications with regard to the possibilities for optimization. The reliability of such screening is enhanced considerably if previous information is already available on the substance ("screening via knowledge"). Mechanistic investigations represent a second focus of in vitro methods. The same principle applies here as with "screening via knowledge," i.e., that the real role of in vitro methods lies in the targeted investigation of specific questions based on previous information obtained from in vivo studies. In vitro methods can add major value, however, e.g., by making it possible for tests to be carried out using human specimens, and thus improve the basis for the risk assessment. Quantitative assessments aimed at extrapolating concentrations from in vitro tests to the in vivo situation (doses) are problematic. It is therefore not surprising that the in vitro test systems established so far for

regulatory purposes (e.g., in vitro genotoxicity, phototoxicity, or cardiotoxicity testing) are almost always part of an integrated battery of in vitro/in vivo tests, and the results of in vitro testing are assessed in the sense of a yes/no answer and not in terms of a quantitative assessment of the risk.

New Developments: Microphysiological Systems (MPS)

To overcome some of the limitations of simple 2D culture systems, several approaches are being pursued to make in vitro models more in vivo-like. From simple to more and more advanced, this includes the following:

1. 2D cell lines in static culture condition representing mostly dedifferentiated cells, i.e., most current screening systems
2. Primary cells in static culture which are associated with some in vivo functionality like CYP enzyme activity in the case of primary hepatocytes and which can be improved with (a) extracellular matrices (ECM), e.g., the hepatocyte sandwich model for shorter-term studies, or (b) co-culture of two cell types, e.g., so-called micro-patterned liver models containing hepatocyte and fibroblasts (Khetani et al. 2015)
3. 3D spheroids either in static culture or in a microfluidics system for at least 2 weeks use (Messner et al. 2013)
4. 3D organoids generated by seeding stem or progenitor cells into an ECM, which then differentiate into different cell types and arrange themselves into organ-like structures (Kretzschmar and Clevers 2016)
5. Microfluidic models for longer-term studies in which microfluidic flow mimics in vivo blood flow and shear stress (Marx et al. 2020; Fabre et al. 2020)
6. 3D-bioprinted models whose major goal is to reach an in vivo-like organ structure with the possibility to print blood vessels and which can be kept in either static or microfluidic modes (Mota et al. 2020)

Currently there is no consensus yet about the exact definition of MPS. Sometimes 3D spheroids or static co-cultures of at least two cell types are counted as MPS, that is, any culture design beyond simple 2D. In a narrower sense, an MPS should be composed of several cell types organized into an in vivo-like structure within a relevant ECM and equipped with flow or other mechanical cues. More advanced systems may contain immune system components.

For MPS models to be useful in preclinical testing and to allow better assessment of potential human safety issues than simple cell line models, they need to contain relevant cell types and should be evaluated for characteristic function of the organ they are supposed to model, including expected responses to a list of reference agents. Specific guidance is given for major organs like the liver (Baudy et al. 2020), lung (Ainslie et al. 2019), kidney (Phillips et al. 2020), and gastrointestinal tract (Peters et al. 2020). In the case of the liver, a recent example showed that it is possible to reproduce species-specific toxicities in an MPS format (Jang et al. 2019). Microfluidics systems also allow to combine different organ models in an in vivo-like circuit, which are called body-on-a-chip systems. With a liver-heart-on-a-chip

system with both hepatocytes and cardiomyocytes derived from the same hiPSC line, a clinically relevant drug-drug interaction could be modeled, in which inhibition of biotransformation of an arrhythmogenic to a non-arrhythmogenic drug in the liver MPS leads to arrhythmia induction in the downstream cardiac MPS (https://www.biorxiv.org/content/10.1101/2020.05.24.112771v2).

A challenge for many advanced models, especially of more elaborate 3D structures, is the introduction of blood vessels to allow supply of oxygen and nutrients in an in vivo-like manner. With 3D bioprinting such vessel structures can be integrated into organ models, as reported for a heart model which allowed the printing of contracting cardiac patches and of thicker vascularized tissues (Noor et al. 2019). To be able to print whole organs, this technology must still overcome many challenges, from recreation of the in vivo blood vessel network to producing sufficient amounts of mature cells. But even such small patches which can be printed already may be used for safety testing or developed toward disease models.

Cross-References

- ▶ Examination of Organ Toxicity
- ▶ Importance of Xenobiotic Metabolism: Mechanistic Considerations Relevant for Regulation
- ▶ Integration of Advanced Technologies into Regulatory Toxicology
- ▶ International Regulation of Toxicological Test Procedures
- ▶ Omics in Toxicology
- ▶ Specific Toxicity Tests for Neurotoxicity, Immunotoxicity, Allergy, Irritation, Reprotoxicity, and Carcinogenicity
- ▶ Toxicodynamic Tests

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Integration of Advanced Technologies into Regulatory Toxicology

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Horst Spielmann and Helena Kandarova

Contents

Abstract

During the past decade regulatory toxicology has been changing its general approach, since it is implementing the "Toxicity Testing the 21st Century" (Tox21) vision proposed by the US National Research Council NRC to reduce and replace traditional safety testing in animals by more predictive toxicity data generated with human cell tissues and organs. New the Adverse Outcome Pathway (AOP) concept allows to integrate non-animal data with existing knowledge into computation models, which allow to predict adverse effects in humans and

H. Spielmann (✉)
Institute of Pharmacy, Freie Universität Berlin, Berlin, Germany
e-mail: horst.spielmann@fu-berlin.de

H. Kandarova
Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine SAS, Slovak Academy of Sciences, Bratislava, Slovakia
e-mail: helena.kandarova@savba.sk

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the environment after exposure to hazardous chemicals with sufficient confidence for safety assessment. The OECD is closely collaborating with national regulatory agencies in Europe and the USA on implementing the AOP concept into "Integrated Approaches for Testing and Assessment" (IATA), which allow regulators to assess the results generated with the new AOP approach for specific endpoints. The Tox21 concept, which is relying on advanced technologies, using human stem cells, multiorgan-chips and in silico models will most probably prove that the future of toxicology will be in vitro.

Keywords

Adverse outcome pathway (AOP) · Human cells and tissues · Integrated testing · IATA · In vitro toxicology · Bioprinting · Multiorgan-chip · Stem cells · ESC · HiPSC · Regulatory testing · Tox21 · Virtual organs

Challenges for Regulatory Testing in the Twenty-First Century

Regulators and the general public are facing increasingly complex challenges that require harnessing the best available science and technology on behalf of patients and consumers. Therefore, we need to develop new tools, standards, and approaches that efficiently and consistently assess the efficacy, quality, performance, and safety of products. However, up to now, the importance of regulatory science has not been sufficiently appreciated and it is underfunded. New scientific discoveries and technologies are not being sufficiently applied to ensure the safety of new chemicals, drugs, and other products to which consumers are likely to be exposed. In addition, members of the public are demanding that greater attention is paid to many more chemicals and products already in commercial use, but the existing testing systems do not have the capacity to deliver the in vivo data required. Thus, we must bring twenty-first-century approaches to twenty-first-century products and problems (Andersen and Krewski 2009).

Most of the toxicological methods used for regulatory assessment still rely on high-dose animal studies and default extrapolation procedures that have remained relatively unchanged for decades, despite the technological revolutions in the biosciences over the past 50 years. The new technologies allow to test tens of thousands of chemicals a year in high-throughput systems, and thousands of chemicals a year in organotypic cultures and low through-put systems. However, we now need to develop better predictive models, in order to identify concerns earlier in the product development process, to reduce time and costs involved in testing, and to reduce the loss of promising biological molecules due to false positive results. We need to modernize the tools used to identify potential risks to consumers who are exposed to drugs, new food additives, and other chemical products.

The challenge today is that the toxicological evaluation of chemicals must take advantage of the on-going revolution in biology and biotechnology. This revolution now permits the study of the effects of chemicals by using cellular components, cells, and tissues – preferably of human origin – rather than whole animals.

The novel regulatory science would take advantage of new tools, including functional genomics, proteomics, metabolomics, high-throughput screening, human-organs-on-a-chip, and systems biology, and could then replace current toxicology assays with tests that incorporate the mechanistic underpinnings of disease and of underlying toxic side-effects. This should allow the development, validation and qualification of preclinical and clinical models that accelerate the evaluation of toxicity during the development of drugs and other chemicals to which humans are exposed. The goals include the development of biomarkers to predict toxicity and screening at-risk human subjects during clinical trials, as well as after new products are made available on the market. The new methods also should enable the rapid screening of the large number of industrial chemicals that have not yet been evaluated under the current testing system, for example, according to the EU chemicals regulation (REACH).

The above-described new technologies enable to generate large datasets (also termed "big data"), which can be utilized in computational toxicology utilizing artificial intelligence tools and machine learning approaches. Currently, the global capacity to test chemicals thoroughly in traditional animal studies would probably not be more than 50–100 chemicals a year. By contrast, the new high-throughput methods developed in the US Environmental Protection Agency (EPA) *Comptox* program (Williams et al. 2017), involving the robotic systems at the National Center for Advancing Translational Sciences (NCATS) at the National Institutes of Health (NIH), could test 30,000 or more chemicals in several hundred functional tests within a year, while by using human cells and systems. This flood of new biological data would drive the development of more-satisfactory, more-predictive computer algorithms that could assist regulatory decision-making. With the addition of data from new human "multi-organ- chip" technologies (*see below*), the regulatory relevance of the data from the high-throughput systems (HTS) could be further refined (Rowan and Spielmann 2019).

Adapting Toxicity Testing to the Challenges of the Twenty-First Century in Europe

To adapt toxicity testing to progress in the life sciences and to end toxicity testing in animals, in the 1980s, various government institutions of the European Commission (EC) and EU Member States promoted and funded the development and validation of in vitro toxicity tests, which were accepted at the international level by the OECD in the early 2000s. Initially, the research activities in Europe were stimulated by the requirements of the EU Cosmetics Directive (EC 2009) and were aimed at ending the suffering of experimental animals in safety tests for cosmetics, and especially in local toxicity tests on the skin and eye. The funding of research in the *Alternative Testing Strategies Programme* of the 6th (FP6) and 7th (FP7) EU Framework Programmes of the Research and Innovation Directorate General of the EC was quite successful, since, for this specific field of toxicology, *in vitro* tests were developed, validated, and accepted by regulators, and the full ban on animal testing

for cosmetic products manufactured or marketed within the EU finally came into forced on 11 March 2013 (EC 2013). Although this was a unique success story, and represented a breakthrough from the scientific, regulatory, and ethical points of view, which was acknowledged around the world, those in vitro toxicity tests were based on the progress with in vitro culture techniques achieved in the twentieth century.

To speed up the change to challenges of the twenty-first century in collaboration with the cosmetics industry the EU FP7 multi-center SEURAT-1 project was established to replace repeat-dose systemic toxicity testing in vivo in animals. In addition, the EU launched another FP7 project, Accelerate (XLR8), to implement the transition to a toxicity pathway-based paradigm for chemical safety assessment, a concept proposed in 2007 by the US National Research Council (NRC) report, *Toxicity Testing in the twenty-first Century: A Vision and a Strategy* (NRC 2007).

The US Vision Toxicity Testing in the Twenty-First Century (Tox21) (US NRC 2007)

The new concept for a toxicity testing paradigm relies mainly on understanding "toxicity pathways" – the cellular response pathways that can result in adverse health effects when sufficiently perturbed (NRC 2007; Andersen and Krewski 2009; Krewski et al. 2010). In the new approach biological significant alterations are evaluated without relying on animal studies. In addition, "targeted testing" had to be conducted, to clarify and refine information from toxicity pathway tests for chemical risk assessments. Therefore, targeted testing in animals will become less necessary, as better systems are developed to understand how chemicals are metabolized in the human body, when applying only tests in cells and tissues. Testing in animals may then be phased out in the next 10–15 years, provided that more resources are devoted to improving regulatory toxicology.

A toxicity pathway refers to a chemically induced chain of events at the cellular level that may ultimately lead to an adverse effect such as tumor formation. Such pathways ordinarily coordinate normal processes, such as hormone signaling or gene expression. For example, a protein that, upon chemical binding, blocks or amplifies the signaling of a specific receptor could alter the pathway's normal function and induce a "pathway perturbation." Dose-response and extrapolation modeling will permit the translation of cellular tests to exposed humans. Specifically, the modeling will estimate exposures that would lead to significant perturbations of toxicity pathways, observed in cellular tests.

The Adverse Outcome Pathway (AOP) Concept

The **a**dverse **o**utcome **p**athway (AOP) concept was proposed as essential element of the Tox21 vision by the US Environment Protection Agency (EPA) , which defined that an AOP is a sequence of key events linking a **m**olecular **i**nitiating **e**vent (MIE) to an **a**dverse **o**utcome (AO) through different levels of biological organization

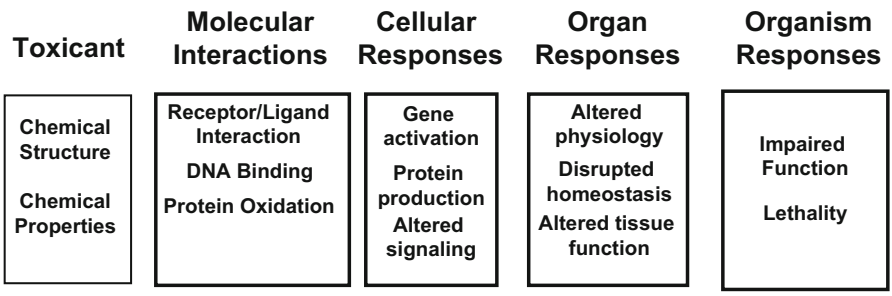

Fig. 1 The adverse outcome pathway (AOP) concept

(Ankley et al. 2010). AOPs span multiple levels of biological organization, and the AO can be at the level of the individual organism, population or ecosystem. Each AOP is a set of chemicals, biochemical, cellular, or physiological responses, which characterize the biological effects cascade resulting from a specific exposure (Ives et al. 2017). The key events in an AOP should both be definable and make sense from a physiological and biochemical perspective. By using the AOP approach, it is possible to identify endpoints of regulatory concern and to ask which toxicity mechanisms are most likely to lead to these outcomes. AOPs are the central element of a toxicological knowledge framework being built to support chemical risk assessment based on mechanistic reasoning.

Meanwhile, the AOP concept has been accepted by the international scientific community, and the OECD launched a new program on the development of **AOPs** in 2013 (OECD 2013). Consequently, the OECD requires that the AOP concept should be considered when new toxicity tests are introduced, or existing ones are updated (OECD 2017a, b). The AOP **k**nowledge **b**ase (AOP KB), set up by the OECD, is a formal Internet-based repository (http://aopkb.org) for information on AOPs. The content of the AOP KB, which contained 284 AOPs in March 2020, continues to evolve, as more information is gained on AOPs, their **k**ey **e**vents (KEs) and **k**ey **e**vent **r**elationships (KERs).

Approaches such as the development of AOPs and the identification of **m**odes **o**f **a**ction (MoA), together with the use of use **i**ntegrated **a**pproaches to **t**esting and **a**ssessment (IATA) (OECD 2017b) as the means of combining multiple lines of evidence, are seen as the fundamental pathway to the hazard identification and characterization of a chemical. MoAs and AOPs are conceptually similar: MoAs include the chemical specific kinetic processes of **A**bsorption, **D**istribution, **M**etabolism and **E**limination (ADME), and describe the mechanism of action of the chemical in the human body, whereas AOPs focus on nonchemical specific biological pathways starting with a **m**olecular **i**nitiating **e**vent MIE (e.g., binding to an enzyme), resulting in perturbations (KEs) leading to an AO at the organism level as outlined in Fig. 1.

An AOP is a conceptual framework that links a molecular-level initiating event with adverse effects relevant for risk assessment. Each AOP consists of a set of chemicals, biochemical, cellular, and physiological responses, which characterize

the biological effects cascade resulting from a specific toxic insult (Ankley et al. 2010). AOPs span multiple levels of biological organization. AOPs often start out being depicted as linear processes; however, the amount of detail and linearity characterizing the pathway between a molecular initiating event and an adverse outcome within an AOP can vary substantially, both as a function of existing knowledge and assessment needs (OECD 2017a).

Integrated Approaches to Testing and Assessment (IATA)

Complex endpoints cannot be predicted by a single stand-alone non-animal test as it will never be possible to reproduce a whole organism, mainly due to the lack of kinetic relationships and cross-talk among cells, tissues, and organs (Rowan and Spielmann 2019). It is instead necessary to use **i**ntegrated **a**pproaches to **t**esting and **a**ssessment (IATA) based on a **w**eight-**o**f-**e**vidence (WoE) approach, where information and evidence from a battery of tests can be incorporated (OECD 2017b). Data can then be integrated by means of modeling. This will lead to a shift toward the use of more human data in terms of biologically significant perturbations in key toxicity pathways.

IATA are scientific approaches to hazard to risk characterization, based on an integrated analysis of existing information coupled with new information by using testing strategies. IATA can include a combination of methods and integrating results from one or many methodological approaches ranging from flexible to rule based ones, so-called defined approaches. IATA should ideally be based on knowledge of the MoA by which chemicals induce their toxicity. Such information is, of course, quite often missing for complex endpoints, for example, carcinogenicity or developmental toxicity. This approach has been used successfully. to partially replace skin sensitization testing in animals: the first three in vitro AOP-based OECD TGs have been adopted covering the skin sensitization AOPs for protein binding (TG 442C; OECD 2015a), as well as for keratinocyte activation (TG 442D; OECD 2015b) and dendritic cell activation (TG 442E; OECD 2016).

New Technologies

During the last 30 years significant increase in knowledge and technology allows us today to conduct safety testing on human cells and on 3D tissue models and organoids rather than on animal models.

The first important step was the discovery and application of stem cells, in particular of embryonic stem cells (ESC) and of adult induced pluripotent stem cells (iPSC) from both animals and humans. At the same time perfused human 3D cell culture organ culture models, multiorgan-chips (MOC), were developed to model human diseases and also to study beneficial and adverse effects of drugs and other chemicals on human tissues and organs. Moreover, quite independently computer based virtual models of organ system have been developed, which

incorporate biological structure and extend the data from in vitro toxicity testing to a higher level of biological organization.

Advancing toxicology in the twenty-first century by applying the new technologies with human tissues – stem cells, perfused organ chips, 3D-bioprinting, virtual models, and use of artificial intelligence (AI) – will be highlighted in this section.

Stem Cells as Advanced Tools in Predictive Toxicology

Stem cells have the ability to differentiate along different lineages and the ability to renew themselves. Stem cells are broadly classified into embryonic stem cells (ESCs) found in the embryo, stem cells isolated from adult tissues, and induced pluripotent stem cells (iPSCs). Along with self-renewal capacity, ESCs are pluripotent cells with the ability to differentiate into three embryonic germ cell layers. Almost 30 years ago, we developed the first stem cell-based toxicity test, the mouse embryonic stem cell test (mEST) (Laschinski et al. 1991), which has become most popular after it was successfully validated by ECVAM (Genschow et al. 2002) and we provided a robust protocol (Seiler and Spielmann 2011). Meanwhile, many variants of the EST have been developed and high-throughput-screening (HTP) variants are used during preclinical drug development.

The most apparent advantage of using hESCs instead of mESCs is to limit the possibility of false negatives that may arise due to species-specific differences. The advanced versions of the humanized hEST are using reporter genes and other biomarkers as endpoints for embryotoxicity. In the US ToxCast program the commercial hiPSC high-throughput assay (Stemina STM) that predicts the developmental toxicity potential based on changes in cellular metabolism following chemical exposure (Palmer et al. 2013, 2017), 1065 ToxCast phase I and II chemicals were screened in single-concentration or concentration-response for the targeted biomarker (ratio of ornithine to cystine secreted or consumed from the media). The encouraging results of this extensive study support the application of the Stemina STM platform for predictive toxicology and further demonstrate its value in ToxCast as a novel resource that can generate testable hypotheses aimed at characterizing potential pathways for teratogenicity and HTS prioritization of environmental chemicals for an exposure-based assessment of developmental hazard (Zurlinden et al. 2020).

Ethical issues of obtaining hESCs from human pregnacies have led to the development of hiPSCs generated from mature somatic cells and reprogrammed to a pluripotent state. hiPSCs possess properties of self-renewal and differentiation into many types of cell lineage that are similar to hESCs. In fact, owing to their ability to differentiate into all the lineages of the human body, including germ cells, stem cells, and, in particular, hiPSC can be utilized for the assessment, in vitro, of embryonic, developmental, reproductive, organ, and functional toxicities, relevant to human physiology, without employing live animal tests and with the possibility of high throughput applications. Noteworthy, patient-derived disease-specific hiPSCs with genetic background sensitive to disease pathology could provide evidence to understand disease mechanisms for developing and testing compounds. Although

variations in differentiation efficiency of various hESC lines may induce significant variability of experimental toxicity data, hESCs can help develop more reliable toxicity testing. Additional significant advantages in using human hESCs are unlimited self-renewal capabilities and differentiation into a variety of specialized cell types. Thus, stem cell toxicology will tremendously assist in the toxicological evaluation of the increasing number of synthetic chemicals that we are exposed to, of which toxicity information is limited.

Human-on-a-Chip (Multiorgan-Chip) Technology Applied to Toxicity Testing

Pressures to change from the use of traditional animal models to novel technologies arise from their limited value for predicting human health effects and from animal welfare considerations (Andersen and Krewski 2009). This change depends on the availability of human organ models combined with the use of new technologies in the field of omics and systems biology, as well as respective evaluation strategies. Ideally, this requires an appropriate in vitro model for each organ system.

In this context, it is important to consider combining individual organ models into systems. The miniaturization of such systems on the smallest possible chip-based scale is envisaged, to minimize the demand for human tissue and to match with the high-throughput needs of industry (Huh et al. 2011; Esch et al. 2015). A multiorgan-chip technology has been developed, based on a self-contained smartphone size chip format, in a project funded by the German Ministry for Research and Technology (BMBF) (Marx et al. 2016, 2020). An integrated micro-pump supports microcirculation for 28 days under dynamic perfusion conditions. The inclusion of human organ equivalents for liver, intestine, kidney, and skin allowed ADME and toxicity (ADMET) testing in a four-organ-chip. The system holds promise for developing disease models for preclinical efficacy and toxicity testing of new drugs. An encouraging example is a human microfluidic two-organ-chip model of pancreatic islet micro-tissues and liver spheroids, which maintained a functional feedback loop between the liver and the insulin-secreting islet micro-tissues for up to 15 days in an insulin-free medium (Bauer et al. 2018), which is a promising simulation of human type 2 diabetes mellitus.

It has been hypothesized that exposure of in vitro assembled premature iPSC-derived organoids to the physiological environment of a **m**icro-**p**hysiological **s**ystem (MPS), such as perfusion, shear stress, electrical stimulation, and organoid cross talk in interconnected arrangements, might constitute the missing step for their final and complete in vitro differentiation. The final aim is to combine different "organoids" to generate a human-on-a-chip, an approach that would allow studies of complex physiological organ interactions. The recent advances in the area of induced pluripotent stem cells (hiPSCs, Ramme et al. 2019) provide a range of possibilities that include cellular studies of individuals with different genetic backgrounds, for example, human disease models. However, throughput remains a significant limitation and there will continue to be a need for emphasis on "fit-for-purpose" assays.

Since 2012, the NIH and the US Food and Drug Administration (FDA) have been funding a major multi-center program for development of a technology platform that

will mimic human physiological systems in the laboratory, by using an array of integrated, interchangeable engineered human tissue constructs — "a human-body-on-a-chip" (NIH 2012). The program, which is coordinated by the National Center for Advancing Translational Sciences (NCATS), intends to combine the technologies to create a microfluidic platform that can incorporate up to 10 individual engineered human micro-physiological organ system modules in an interacting circuit. The goal of the program is to create a versatile platform capable of accurately predicting drug and vaccine efficacy, toxicity, and pharmacokinetics in preclinical testing.

Results obtained with chips and microfluids systems indicate that static and dynamic conditions of in vitro cultures may provide significantly different predictions for some endpoints, but they also bring different view on the possibility of future in vitro assessment of the absorption, distribution, metabolization and excretion of drugs and xenobiotics. The developers of chips and microfluids systems anticipate that once these systems get validated for specific applications in toxicology, they will also be swiftly adopted into the pre-clinical stages of drug development. The new culture systems will be adaptable to the integration of future technologies, for example, advances in stem cell culture, 3D-bioprinting, and into personalized medicine using individual patient-derived tissue (Marx et al. 2020; Marrella et al. 2020).

3D-Bioprinting

3D bio-printing is a new type of tissue engineering technology that is expanding hand in hand with advances in material engineering and bio-polymer chemistry. In this technology, cell-laden biomaterials are used as "bio-inks" and raw materials. Compared to the classic tissue engineering that provided highly standardized in vitro skin and epithelial models, 3D bioprinting allows to produce highly organized 3D tissue models, which are physiologically and morphologically similar to their in vivo biological counterparts (Weinhart et al. 2019). In addition to vascularize the 3D-printed in vitro models, in combination with microfluidic and micro-physiological systems it offers a very promising platform for precisely monitored, long-term toxicity studies. It is expected that the 3D bioprinting technology will be used to construct tissues and organs with complex responses and will especially be applied in safety and efficacy studies of novel drugs.

The use of 3D bio-printed tissues and organs will also provide new approaches for high-throughput toxicity testing that will improve the prediction of human responses to chemical and drugs. In order to implement these technologies into the regulatory framework, it will be necessary to adapt the concept of "open-source" models and to integrate them it into existing TGs. The standardization and validation of these systems will be challenging but they are inevitable.

Virtual Organ Models

Cell-agent based models are useful for modeling developmental toxicity by virtue of their ability to accept data on many linked components and implement a morphogenetic series of events. These data may be simulated (e.g., what is the

effect of localized cell death on the system?) or data derived from in vitro studies. In the latter case, perturbed parameters are introduced as simple lesions or combinations of lesions identified from the data, where the assay features have been annotated and mapped to a pathway or cellular process implemented in the virtual model. Whereas in the EPA ToxCast program predictive models are built with computer-assisted mapping of chemical-assay data to chemical endpoint effects (Judson et al. 2010), the virtual tissue models incorporate biological structure, and thus extend the in vitro data to a higher level of biological organization. A developing system can be modeled and perturbed "virtually" with toxicological data, then the predictions on growth and development can be mapped against real experimental findings.

The goal of the US "Virtual Liver" project is to develop models for predicting liver injury due to chronic chemical exposure, by simulating the dynamics of perturbed molecular pathways, their linkage with adaptive or adverse processes leading to alterations of cell state, and integration of the responses into a physiological tissue model. When completed, the Virtual Liver Web portal and accompanying query tools will provide a framework for the incorporation of mechanistic information on hepatic toxicity pathways, and for characterizing interactions spatially and across the various cells types that comprise liver tissue. The German BMBF funded Virtual Liver Project focuses on the establishment of a 3D model of the liver that correctly recapitulates alterations of the complex micro-architecture, both in response to, and during regeneration from, chemically induced liver damage https://fair-dom.org/partners/virtual-liver-network-vln/. The long-term goal will be to integrate intracellular mechanisms into each cell of the model, as many of the critical intracellular key mechanisms still need to be elucidated.

The US EPA program, the Virtual Embryo Project (v-Embryo™ https://www.ehd.org/virtual-human-embryo/), is a computational framework for developmental toxicity, focused on the predictive toxicology of children's health and developmental defects following prenatal exposure to environmental chemicals. The research is motivated by scientific principles in systems biology, as a framework for the generation, assessment, and evaluation of data, tools, and approaches in computational toxicology. The long-term objectives are: to determine the specificity and sensitivity of biological pathways relevant to human developmental health and disease; to predict and understand key events during embryogenesis leading to adverse fetal outcomes; and to assess the impacts of prenatal exposure to chemicals at various stages of development and scales of biological organization.

Artificial Intelligence (AI) and Machine Learning

High amounts of newly generated in vitro data present an opportunity for using artificial intelligence (AI) and machine learning to improve the knowledge on toxicity pathways and offer a broader insight into the safety assessment of chemicals

and mixtures. The upcoming decade will be certainly an era of "big data" requiring novel approaches to traditional methods of data analysis. This will present both a challenge and an opportunity for toxicologists and regulators. While in the twentieth century, the community only slowly accepted in silico as a discipline of toxicology, in the twenty-first century, we will be more and more exposed to the new QSARs, artificial intelligence and machine learning methods leveraging neural networks (Tang et al. 2019).

These technologies are very promising and already in use by the pharmaceutical industry and some regulatory agencies in the USA. They will reduce the use in vivo and in vitro experiments due to the predictions based on computational ("in silico") modeling, which is risk-free, low-costly, and high-throughput. On the other hand, it will be a challenge to introduce these and more complex systems into the non-expert community.

The Future of Toxicology Is in Vitro

The process of validation of new approaches needs to be reconsidered in terms of efficiency and time to completion (Rowan and Spielmann 2019). In particular, the scientific community needs to understand that if it is possible for advanced non-animal methods to meet some or all regulatory needs. Furthermore, the fate of the animal testing in this transitional phase toward IATA is unclear.

Therefore, it is not surprising, but encouraging, that by the end of 2017, in the USA the FDA and the NIH had published new roadmaps for toxicity testing, which were based on the new principles of safety testing without animals by employing the novel molecular and computational techniques, for example, the *FDA Predictive Toxicology Roadmap* (FDA 2017) and the ICCVAM *Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States* (ICCVAM 2018). In this context, the FDA makes the most pragmatic proposal, and suggests that "Rather than validation, an approach we frequently take for biological (and toxicological) models and assays is qualification. Within the stated context of use, qualification is a conclusion that the results of an assessment using the model or assay can be relied on to have a specific interpretation and application in product development and regulatory decision-making" (FDA 2017).

It is also very encouraging that, early in the twenty-first century, the US regulatory agencies are giving the "long sought goal of refining, reducing, and replacing testing on animals" the high priority that it deserves, both for scientific and for animal welfare reasons, in accordance with the hopes expressed 60 years ago by the pioneers of the Three Rs concept, William Russell and Rex Burch (1959).

In conclusion, twenty-first-century technologies are providing multi-dimensional human data at the molecular and cellular level that will significantly advance regulatory toxicology.

Cross-References

- ▶ Aims and Mission of Regulatory Toxicology
- ▶ Computer-Based Prediction Models in Regulatory Toxicology
- ▶ Examination of Organ Toxicity
- ▶ Institutionalized Participation in Regulatory Toxicology
- ▶ Limit Values and Guideline Values in Regulatory Toxicology
- ▶ National and International Collaboration in Regulatory Toxicology
- ▶ Omics in Toxicology
- ▶ REACH and CLP: Its Role in Regulatory Toxicology
- ▶ Toxicity Testing In Vitro: Regulatory Aspects

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Computer-Based Prediction Models in Regulatory Toxicology

14

Thomas Steger-Hartmann and Scott Boyer

Contents

Abstract

The increasing regulatory safety demands for the submission and registration of chemicals, pesticides, or pharmaceuticals as well as tightening animal protection legislation have exacerbated the dilemma of regulatory toxicology, where on the one hand the required scientific contributions for the protection of workers, consumers, or patients are constantly augmented while on the other hand the number of experimental animal studies should be reduced.

One way to resolve this dilemma could be the use of computer-assisted systems to predict toxic effects. These so-called "in silico" tools have experienced improvements in their performance and predictive power over the past three

T. Steger-Hartmann (✉)
Head of Investigational Toxicology, Bayer AG, Pharmaceuticals, Berlin, Germany
e-mail: thomas.steger-hartmann@bayer.com

S. Boyer
Synapse Managers, Barcelona, Spain
e-mail: sboyer@synapse-managers.com

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decades. They are therefore able to contribute to hazard identification and risk assessment at least for some toxicological endpoints. However, knowledge of how these systems work, the importance of the underlying data quality, and their respective limitations are prerequisites for a sensible application.

Introduction into the Principles of Predictive Systems

Providing a current overview of available predictive systems is not a meaningful exercise for a textbook since the pace of development would make such a list outdated at the time of print. Therefore, this chapter will rather focus on the illustration of the basic principles of predictive tools in toxicology, give an overview of the current fields of application, and provide an outlook on expected future developments.

Comparing a new substance of unknown toxicity to compounds with reasonable structural or biological similarities for which useful toxicological information exists in the scientific literature represents a significant effort. Searching publications to compare an unknown compound with a structure which has been toxicologically characterized would require knowing all the chemical and trivial names and synonyms of the old and new compound as well as subsequently carefully curating the collected literature information. For these reasons, the classical literature search is often replaced by database searches, especially if these databases allow for structure or substructure (i.e., a smaller moiety contained in the larger chemical structure) queries. The structural comparison is performed in the context of the toxicological endpoint of interest. Once the user is convinced that the chemical structures retrieved in the database search is sufficiently similar to the new compound, they can *read-across* any findings from the existing compounds and data in the database and thereby come to conclusions regarding potential toxicological issues of the new compound. The number of publically or commercially available toxicology databases is constantly growing. Particularly in the field of pharmaceuticals, endpoint-specific databases have evolved (Luo et al. 2017); however, the possibility for structural searches is still a rare feature. A list of the most relevant databases is included at the end of this chapter.

With the accumulation of toxicological data around a specific endpoint, it has become possible to generate relationships between chemical structure and toxicological effect. Structure activity relationships (SARs) can be performed by correlating both the chemical structures with the toxicological data. The resulting SAR can then be coded into a computer program which is able to read in a new chemical structure, recognize the presence of structures or substructures for which toxicological data is available. The results are then presented to the user with an estimate of the toxicity of the new structure. In principle, there are two different approaches for constructing SAR: expert system and quantitative structural activity relationships, though hybrid systems combining the two underlying methods have been developed over the recent decade.

Expert systems, the most commonly used of which is DEREK (*D*eductive *E*stimation of *R*isk from *E*xisting *K*nowledge) from Lhasa Ltd., analyze the compound under investigation for substructures with toxicological relevance. Usually an expert system provides the information (rule) why a specific (sub-)structure causes a warning ("alert") such as "acid halides may cause skin sensitization." This offers the opportunity to the user to assess the plausibility of the alert. Many of the well-established alerts have been made available in the public domain, i.e., a user may screen a compound of interest via internet-based tools (Sushko et al. 2012). In the original versions of expert system, such alerts related almost exclusively to the graphical comparison of the two-dimensional (2-D) chemical structure, i.e., conformational changes of a molecule are ignored. Other properties such as the physicochemical nature of the compound, which can significantly influence the toxicological properties of the chemical under investigation, are not necessarily accounted for, though they may influence the occurrence of toxicity. This shall be illustrated for the prediction of the endpoint skin sensitization. For a chemical to cause skin sensitization, a particular reactivity with proteins is required to elicit the immune response. To identify the chemical reactivity knowing the 2-D structure of the compound often suffices. However, the reaction with skin proteins can only occur if the compound shows penetration into the skin. Skin absorption is a parameter which cannot always be derived from the 2-D structure of the chemical, but rather requires knowledge of additional properties ("descriptors") to determine the lipophilicity or the octanol-water partition coefficient of the compound which correlate with skin penetration. Advanced Expert systems thus calculate the parameters for reactivity and absorption individually and then combine these values with a prediction of the chemical reactivity using "if. . .then" rules.

An alternative approach to Expert systems is the *quantitative structure activity relationship* (QSAR). QSAR systems (represented in commercial systems like Leadscope, Sarah Nexus [Lhasa Ltd.], MultiCASE, TOPKAT [BIOVIA], or freely available systems implemented in the OECD Toolbox) use chemical, physicochemical properties, or other descriptors such as molecular fragments, indices of electron density, or polarity and correlate them with either the binary outcome of an experiment (positive or negative such as "mutagenic" or "non-mutagenic") or a quantitative (or continuous) outcome, e.g., number of revertants in an Ames mutagenicity assay, inhibition constants of an enzyme, receptor, or ion channel (Tropsha 2010). In its simplest form, the relationship can be calculated based on a linear or nonlinear regression. The quality of the correlation can then be assessed by calculating the partial least squares regression. In case more descriptors are used to characterize compounds, multivariate statistics are applied. The statistical methods to establish these correlations are numerous and beyond the scope of this chapter. The choice of both the descriptors and the statistical method to correlate toxicological outcome with the chemical structure is dependent on the nature of the data set and size as well as the toxicological endpoint (how many details of the toxic effects shall be modeled). One way to characterize the chemical structure is the use of so-called fingerprints which are able to cover several fragments contained in a structure.

Depending on the available computer power also the 3-D conformation, which can be of relevance if the toxic effect is caused by binding to receptors or enzymes, can be included. To calculate the correlation between the fingerprints and the biological effects, powerful statistical machine learning algorithms (neural network, support vector machine, random forest) are applied. The drawback of these methods is that they sometimes suffer from a lack of interpretability, either because the underlying mechanisms of toxicity cannot easily be identified for endpoints such as hepatotoxicity, or the interpretability is hindered by the lack of an ability within the machine learning algorithm to clearly identify what structural features or properties of the molecules are contributing to the toxicity endpoint (Hasselgren et al. 2013). To allow for interpretation of QSAR models, the dataset that has been used to "train" the model should always be made available in order to be able to assess in how far a new compound is covered by the original training set (the "applicability domain," discussed below). This is critical for the interpretation of the outcome of the model as discussed in the following sections.

Assessment of Predictive Systems

The prediction quality of in silico tools can be described with similar statistical parameters also established for medical diagnostic procedures. The predominant criteria are *sensitivity*, *specificity*, and *accuracy*. A definition for these parameters and further assessment criteria are provided in Table 1.

Definitions

Sensitivity (true positive rate): The ability to predict positives when they actually exist

Specificity (true negative rate): The ability to predict negatives when they actually exist

Positive predictive value: The ability of the predictive system to distinguish between true positives and false positives

Negative predictive value: The ability of the predictive system to distinguish between true negatives and false negatives

Overall (observed) accuracy (concordance): The total number of correct predictions divided by all examples

In order to determine sensitivity and specificity, a certain amount of compounds with known toxicological properties, usually those which have not been used in the development of the model ("validation set," which is not included in the "training set") are predicted in a given model or system. The predicted effects are then compared to the effects observed in the actual experiment. The simple example of a two-class categorical model is shown in Table 1 and can be applied to binary models of the type "toxic" or "nontoxic," i.e., are particularly relevant for hazard

Table 1 Confusion matrix for a categorical two-class model

		Experimental outcome		
		Test positive	Test negative	
Prediction outcome	Predicted positive	**True positive (TP)**	**False positive (FP)**	Positive predictive value
				TP/(TP + FP)
	Predicted negative	**False negative (FN)**	**True negative (TN)**	Negative predictive value
				TN/(FN + TN)
		Sensitivity	Specificity	
		TP/(TP + FN)	TN/(FP + TN)	

identification (e.g., prediction of mutagenicity) or where cut-off values are used to distinguish between toxic and nontoxic. It has to be noted though that the calculation of these parameters is always dependent both on the data set used to develop the model as well as on the compounds selected for prediction, i.e., the parameters do not represent absolute or constant quality indicators for a specific model (Simon-Hettich et al. 2006). Even for well-established endpoints such as mutagenicity, these parameters may vary widely, but as a rule of thumb values above 80% indicate good model performance (Sutter et al. 2013).

As noted above, many toxicological-relevant endpoints are recorded as continuous variables, e.g., inhibition of a receptor or a cellular transporter. In these cases, the confusion matrix cannot be used, but rather a QSAR's predictive performance has to be evaluated by an analysis of correlation between the predicted values and observed values for compounds which are not included in the training set. A common parameter for assessing the quality of the QSAR is the root mean squared error (RMSE), i.e., the standard deviation of the residuals between predicted and measured toxicity. RMSE evaluation has become a quality-preferred parameter for QSARs since it relates the prediction error, as an expression of RMSE, with the experimental error which is frequently expressed as RMSE as well (Alexander et al. 2015).

Regardless of the data type or evaluation method, the results of such comparisons are not absolute, but again rather dependent on the training set.

The selection of the structures for building the expert or QSAR system determines the quality of the predictions. Many QSAR systems are built on data from industrial chemicals or pesticides as abundant data sets are available in the public domain for these compound classes. As a consequence, these systems are predictive primarily for a similar so-called chemical space, i.e., for structures which have similar chemical properties. Predictions for other areas of chemical use, especially for pharmaceuticals tend to perform worse in these systems for above-mentioned parameters. Comparisons of industrial chemicals, pesticides, pharmaceuticals, and food-related chemicals show that these various classes are both chemically and toxicologically distinct and should probably be modeled separately. As a consequence, it is now common practice to provide information on the prediction limits of the model or

system, otherwise known as the "domain of applicability." The domain of applicability usually represents the chemical domain for which robust prediction can be made based on the selected training set. A common approach to assess the domain of applicability lies in the analysis of chemical similarity of the compound under investigation with the list of compounds used to build the system. Alternatively, a system could be split up on several "local" models, each of which represent a distinct chemical class for which it can be applied (e.g., prediction of ecotoxicity of different classes of environmental pollutants in silico tool ECOSAR provided by the US Environmental Protection Agency [EPA]).

Even if the chemical space for a specific model is wide and the underlying training set is of good quality, a perfect concordance between prediction and the experimental result should not be expected. Several factors prevent obtaining a perfectly predictive SAR or QSAR system. Paramount among these factors is the toxicological endpoint itself. Most endpoints are observations of a complex series of biochemical and biological events that culminate in an observed toxicity. Many toxicological observations can be caused by more than one mechanism and thus expecting a single chemical structure-toxicity relationship to reflect these various mechanisms is expecting too much from both the method and the data. The second layer of complexity in toxicological data is data reproducibility. Inter-laboratory variability means that even well-known and mechanistically simple (relatively speaking) endpoints like the Ames mutagenicity assay have an imperfect concordance and thus a "positive" in one laboratory may be "negative" in another (McCann et al. 1984). While perfectly understandable, variability in the underlying data means that SAR or QSAR models built with these data will possess inherent limitations in prediction. Regarding the prediction of human safety, the translatability of preclinical toxicological outcomes to human effects is also an issue. A comparison of effects reported in animal studies with those observed in humans results in a concordance which ranges around 60%, where some endpoints show a better predictivity than others (Olson et al. 2000). It should be noted that a perfect concordance between preclinical toxicological outcomes and human outcomes may not be possible due to the fact that many substances which are found to have significant toxicity in preclinical models (animals, in particular) are never administered to humans and thus the completeness of the preclinical-clinical comparison datasets may always pose a validation challenge. Thus, predictive systems built on data from animal studies will not be able to achieve a better prediction of human effects than the animal studies themselves. However, higher concordance rates should be achievable for specific human endpoints if mechanistic information is integrated into the models and the specific mechanisms are linked to human outcomes.

Data quality assessment is one of the cornerstones of good SAR and QSAR modeling practice. Whereas expert systems often reference the scientific literature and thus allow the user to assess the quality of the data used, such a quality assessment (was the study performed according to international guidelines, according to GLP regulations, was the study published in a peer reviewed journal, etc.?) is often difficult to evaluate in QSAR systems because the supporting quality

information of the training set is only rarely made available. Data quality assessment schemes have been developed for regulatory context to assess both relevance and reliability of experimental data (Klimisch et al. 1997; Kaltenhäuser et al. 2017). These schemes also provide a systematic way to combine data of similar quality for the development of predictive tools. In addition to questions regarding data quality, often there are issues with data completeness as well. The construction of toxicological QSARs relies mainly on data from experiments for which a toxic effect was recorded. The unfortunate consequence of this is that negative effects, i.e., the absence of any effect, are only rarely published in the scientific literature. As a consequence QSAR training data sets collected from the scientific literature often show an overabundance of compounds which cause toxicity even though in the experimental system actually very few positive compounds were observed, i.e., the training set if often unbalanced. For example, in the Registry of Toxic Effects of Chemical Substances (RTECS), which was initially curated by the US National Institute for Occupational Safety and Health (NIOSH), the endpoint mutagenicity contains only compounds which show a positive effect in the different assays for genotoxicity. Negative results, i.e., nonmutagenic outcomes are not reported. Systems built on such databases risk overpredicting the effects under investigation. Such systems usually have a high sensitivity at the cost of a low specificity, i.e., many compounds are forecast to be mutagenic, even though they might contain certain properties (e.g., groups causing steric hindrance) which prohibit the activation to a mutagenic intermediate. Such compounds might have been tested but are not published and therefore their negative effects are not reflected in the QSAR. This situation is gradually improving, as the scientific community begins to see the utility of publishing and sharing data showing that a certain compound has been characterized for a toxicological effect and been found to be negative.

In order to resolve some of these issues, initiatives or consortia which gather unpublished data for the construction of new predictive tools have been formed. The advantage of such initiatives is the possibility of sharing unpublished, proprietary data and structures in a controlled way. This approach is particularly important for the pharmaceutical industry. In this field, only very few toxicological studies are published in comparison to the number of compounds that is actually experimentally evaluated, but fail to become drugs for various reasons. As an example, the eTOX project ("electronic toxicity") performed under the European Innovative Medicines Initiative over the last years developed a preclinical drug safety database of unpublished systemic toxicity results of more than 8000 animal studies, which can now be used for read-across or predictive tool development (Steger-Hartmann and Pognan 2017).

Use of Predictive Tools in Regulatory Toxicology

In 2004, the OECD guidance document on principles for the validation of (Q)SAR was adopted (OECD 2004). In 2008, the first version of the OECD QSAR Toolbox was released. The OECD Toolbox is intended to be used in the context of European

industrial chemical assessment and registration REACH (Regulation, Evaluation, Authorization, and Restriction of Chemical Substances) (OECD 2013). The toolbox represents a collection of databases, tools for read-across, and predictive systems. The systems allow for retrieval of structurally similar compounds which have already been assessed or are registered and for which toxicological data are available. Based on these comparisons, the systems provide data for read-across and expert hypotheses on potential effects for the compound without toxicity data.

In an alternative approach, the REACH data available via IUCLID (International Uniform ChemicaL Information Database) was subjected to a complex analysis of chemical fingerprints, similarity measures, and machine learning algorithms to derive prediction systems for six human health hazards (skin sensitization, eye irritation, acute oral toxicity, mutagenicity, skin irritation/corrosion, acute dermal toxicity) (Luechtefeld et al. 2016). The software was subsequently commercialized under the name of REACHAcross™.

In the US-EPA initiative ToxCast, which is part of the Toxicology in the twenty-first century (Tox21) US federal agency collaboration, more than 8000 chemicals (industrial, consumer and environmental chemicals as well as drugs) have been investigated in a multitude of high-throughput assays for different endpoints (gene expression, enzyme inhibition, receptor binding, cell cycle arrest, etc.). The data sets have been made publicly available through the ToxCast Dashboard (https://www.epa.gov/chemical-research/toxcast-dashboard) and can be used for read-across particularly regarding mechanistic hypotheses on chemical actions or for establishing new predictive models, e.g., for endocrine disruption through activity on estrogen receptors. Several predictive models have been developed based on this data set which fulfills many of the criteria discussed above regarding data quality and consistency.

For *cosmetics and toiletries*, the Cosmetics Regulation (replacing the Cosmetics Directive 76/768/EEC) resulted in a complete ban of animal studies for the human risk assessment of cosmetics since 2013, irrespective of the availability of alternative nonanimal methods. While in vitro replacement methods for a number of the endpoints have been developed such as skin and eye irritation or skin sensitization and found international regulatory acceptance, this is not the case for the more complex endpoints such as repeated-dose toxicity or reproductive toxicity. In 2013, the OECD (revised guidance: OECD 2017) proposed a concept to approach these complex endpoints with the help of "adverse outcome pathways" (AOPs), which "delineates the documented, plausible, and testable processes by which a chemical induces molecular perturbations and the associated biological responses which describe how the molecular perturbations cause effects at the subcellular, cellular, tissue, organ, whole animal and population level." The objective of AOPs is to identify the crucial mechanistic steps which lead to the effect observed in vivo. Based on these analyses, replacement methods or predictive tools can be built which represent these mechanisms. Currently, skin sensitization is the only endpoint which has been successfully deconvoluted with the AOP approach into three in vitro assays replacing the in vivo study (local lymph node assay in mice). It has also triggered numerous approaches to combine the in vitro results into prediction models for this

endpoint, though a model with broad regulatory acceptance is yet not available. Despite these efforts in AOPs, a complete replacement of animal studies for cosmetics assessment is not possible within the near future.

Currently, the only case where the exclusive use of in silico tools has achieved regulatory acceptance is the assessment of potentially mutagenic impurities in *pharmaceuticals* (ICH M7(R1) 2017). Impurities may occur in the drug product from degradation of the active pharmaceutical ingredient or as remaining traces of synthetic intermediates. In many cases, these impurities occur in extremely low concentration and/or are unstable or reactive making it impossible to be isolated and tested separately in in vitro or in vivo assays. As long as the chemical structure has been elucidated, the DNA reactive (mutagenic) potential of such an impurity may be assessed with predictive tools (Glowienke and Hasselgren 2010; Sutter et al. 2013). If such an assessment based on in silico tools (usually an expert system and a QSAR methods are applied) does not identify a structural alert for mutagenicity, and the prediction of a lack of mutagenic potential can be supported by expert knowledge (i.e., read-across of pre-existing data), the mutagenicity assessment exclusively based on in silico prediction is usually accepted by the regulatory authority.

Future Perspectives

The last decade has seen the gradual obliteration of the boundaries of classical read-across, trend analysis, and in silico prediction particularly for the purpose of closing data gaps in the field of industrial and consumer chemical safety assessment. It has become evident that the application of predictive tools not only speeds up the process, but may also lead to standardization and a higher reproducibility. On the other hand, it is also obvious that the prediction of in vivo toxicities is still a remote goal, particularly when it comes to predict the exposures at which toxic effect might occur, which is, however, paramount for risk assessment particularly in the pharmaceutical domain.

Four areas of development are identified, which will contribute to the advancement of the tools in the future:

1. Enhanced availability of high-quality standardized and curated data: Besides providing access to large data sets as done by EPA (ToxCast) or ECHA (REACH database), regulatory agencies undertake efforts to standardize toxicity data used for submission. The FDA has required since 2016 that the pivotal preclinical studies are submitted according to the Standard of Exchange of Nonclinical Data (SEND). Industry consortia have been established to also collect and share such data for the purpose of read-across and data mining (e.g., IMI eTransafe; http://www.etransafe.eu/).
2. New approaches to data analyses and visualization: As soon as the more complex data from systemic toxicity studies are used for data mining and development of predictive tools, the classical two-class or regression models will not suffice to fully represent evident or hidden relationships between chemical structure and

toxicity. Neural networks or so-called deep learning approaches might contribute to uncover some additional underlying knowledge. In addition, automated ways to intuitively visualize both the data mined in these large data sets as well as predictive outcome for large data sets are being developed. It is also foreseeable that these new approaches will provide inroads to definitions of "biological similarity" complementary to the chemical similarity which currently still represents the backbone of predictive models.

3. Quantitative adverse outcome pathways (qAOPs) and systems toxicology: The concept of AOPs has contributed to a better mechanistic understanding of certain toxicological processes and has imposed more discipline in parsing the various molecular mechanisms that could contribute to a toxicological outcome. In order to make AOP applicable for risk assessment, the quantitative aspect of pathways is required to predict tipping points, where a key initiating event changes from an adaptive process into toxicity. Systems toxicology, i.e., the integration of classical toxicology with quantitative analysis of molecular and functional changes occurring across multiple levels of biological organization together with technologies for predicting organ or tissue exposure will help facilitate the step from in silico hazard identification to in silico risk assessment.
4. Improved methods to determine applicability domain and uncertainty of prediction: The level of trust in predictive models is largely dependent on the definition of boundaries of what they can predict and what they cannot. Methods for evidence combination which take into account the sources of uncertainty throughout the modelling process (including applicability domain) and express the resulting prediction as a probability while still maintaining transparency regarding the sources of uncertainty in the conclusion will contribute to the improvement and ultimate acceptance of predictive models in risk assessment.

Resources

List of noncommercial toxicological internet data sources including a short description:

CTD (Comparative Toxicogenomics database; a publicly available database focusing on gene expression data that aims to advance understanding about how environmental exposures affect human health. Structure-based searches are not possible): http://ctdbase.org/

COSMOS (toxicological data and information from regulatory submissions and the literature, focusing on chronic toxicity assessment. The database was developed in the framework of the European SEURAT project. Structure-based searches are possible): http://www.cosmostox.eu/what/COSMOSdb/

DrugBank (a chem- and bioinformatics resource supported by the Canadian Institutes of Health Research that combines drug (i.e., chemical, pharmacological and pharmaceutical. Structure-based searches are not possible) data with drug target information): https://www.drugbank.ca/

DSSTox (Distributed Structure-Searchable Toxicity Database; a resource for public chemistry data, including bioassay and physicochemical data maintained by US EPA. Structure-based searches are not possible): https://www.epa.gov/chemical-research/distributed-structure-searchable-toxicity-dsstox-database Chemicals from the DSSTox can be either downloaded as sd files or accessed via the two dashboards:
- ToxCast (EPA's ToxCast data can be queried for assays and chemical names or CAS numbers): https://actor.epa.gov/dashboard/
- Chemistry Dashboard: https://comptox.epa.gov/dashboard

eTOXsys (user interface of the IMI eTOX project containing a sample set of systemic toxicity studies and predictive tools developed in the project): https://etoxsys.eu/etoxsys.v3-demo-bk/dashboard/

IUCLID (REACH study results; a collection of nonconfidential substance data that was submitted to ECHA under the REACH regulation): https://iuclid6.echa.europa.eu/reach-study-results

LiverTox (a database hosted by the US National Library of Medicine providing information about drug-induced liver injury caused by prescription and nonprescription drugs, herbals, and dietary supplements): https://livertox.nih.gov/

Liver Toxicity Knowledge Base (LTKB) (a database hosted by the FDA containing drugs whose potential to cause DILI (Drug-Induced Liver Injury) in humans has been established using the FDA-approved prescription drug labels): https://www.fda.gov/ScienceResearch/BioinformaticsTools/LiverToxicityKnowledgeBase/default.htm

National Toxicology Program

- Tox21 Toolbox (tools for visualizing dose-response curves or profiling activities of the compounds tested within the Tox21 program): https://ntp.niehs.nih.gov/results/tox21/tbox/
- Tox21 Data Browser: https://tripod.nih.gov/tox21/index
- Drug Matrix Database (toxicogenomic reference resource for drugs and environmental chemicals): https://www.niehs.nih.gov/drug_matrix
- TG-GATES database (public toxicogenomics database supported by the National Institutes of Biomedical Innovation, Health and Nutrition containing data on gene expression and detailed toxicity data from rats and the primary cultured hepatocytes of rats and humans): http://toxico.nibiohn.go.jp/english/index.html
- ToxNet (resources for searching databases on toxicology, hazardous chemicals, environmental health, and toxic releases maintained by the US National Library of Medicine. Structure-based searches are not possible): https://toxnet.nlm.nih.gov/newtoxnet/toxline.htm
- Subsets of mutagenicity and reproductive toxicity data (DART) can be found:
  - https://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?GENETOX
  - https://toxnet.nlm.nih.gov/newtoxnet/dart.htm
- Toxic Exposome Database (formerly called T3DB, a database supported by the Canadian Institutes of Health Research with a focus on providing mechanisms of toxicity and target proteins for chemical compounds. Structure-based searches are possible): http://www.t3db.ca/

Selection of Freely Available Software and Tools

ECOSAR: https://www.epa.gov/tsca-screening-tools/ecological-structure-activity-relationships-ecosar-predictive-model
eTOX: http://www.etoxproject.eu/
OECD toolbox: http://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm
ToxAlerts: https://ochem.eu/alerts
VEGAHUB: https://www.vegahub.eu/

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Metabolism Investigations in Drug Development

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Jens Riedel and Alain Krick

Contents

Abstract

Most small drug molecules are metabolized in the liver and other drug-metabolizing organs. This has considerable consequences: The available concentration of the original active compound decreases, and metabolites may possess a

J. Riedel (✉)
Sanofi-Aventis Deutschland GmbH/DMPK/Metabolism & tMSI, Industriepark Höchst, Frankfurt am Main, Germany
e-mail: Jens.Riedel@sanofi.com

A. Krick
Sanofi/Research/DMPK/Metabolism, Chilly Mazarin, France
e-mail: Alain.Krick@sanofi.com

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pharmaceutical or toxic activity. Advanced separation and detection methods are used in drug development to reveal the complete metabolic pattern. Likewise, protein-based biopharmaceuticals tend to get degraded in the living organism by proteinases, or are otherwise modified, yielding potentially toxic products.

The importance of drug metabolism in drug development and for drug safety is discussed, and common investigation techniques described.

Keywords

Biotransformation · Phase I metabolism · Phase II metabolism · Protein degradation · Metabolite analysis · Toxic metabolite · Drug interaction · Metabolite interaction

Introduction

Investigations on the metabolism of drugs are meaningful for two reasons. Firstly, drug metabolites can be pharmacologically active or cause adverse reactions via on-target or off-target interactions. Secondly, potential interactions with other co-medications are linked to metabolic enzymes which can be investigated in suitable in vitro test systems. Both provide predictive information to assess the potential risk for patients before approval of a drug and even before the first clinical trials and thus, contribute to the minimization of risk for healthy subjects and patients in clinical trials. The strategies and approaches assessing the metabolism of small molecule drugs – also known as new chemical entities (NCE) – are well established with defined study packages throughout the discovery and development phase, and with regulatory guidance provided by the authorities. In contrast for the emerging biotherapeutic drugs – also known as new biological entities (NBE) – the current situation is more unclear and therefore is not the focus this book chapter.

Profiles and Structure Elucidation

To properly assess the impact of metabolites on efficacy and safety, it is necessary to have some knowledge of their structure and concentrations in the body.

As a rule of thumb, 70% of drug-related material of an NCE, present either as parent compound or metabolites, excreted via urine and feces or circulating in blood, should be structurally identified in animals and human. These investigations typically employ radiolabeled drugs in order to quantify drug and metabolite concentrations in various biological matrices regardless of the knowledge of the chemical structure. Radionuclides applied are typically carbon-14 [^{14}C] and tritium [^{3}H] due to ease of use, the synthetic accessibility, and the analytical traceability without change of the biological properties of the investigational compound.

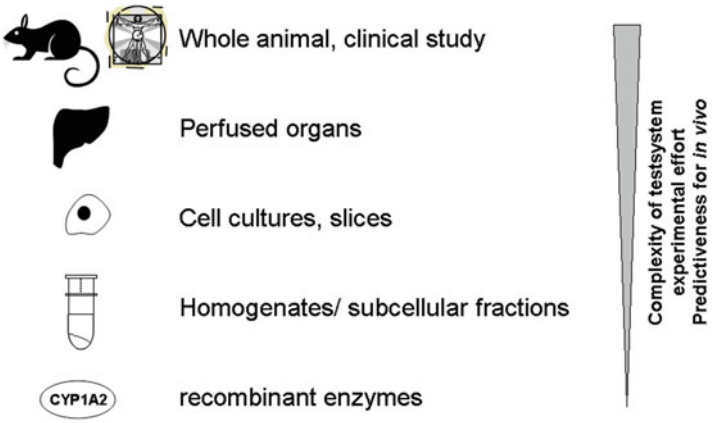

Fig. 1 In vitro-test systems used for metabolism studies in comparison to in vivo models

In Vitro Test Systems

In vitro investigations on metabolism give rise to basic information on the biotransformation of a compound before animal studies or clinical investigations. Drug metabolism is due to biotransformation during transfer into the systemic elimination, e.g., for orally administered compounds, a potential degradation in the gastrointestinal tract and in the gut mucosa, or for inhaled compounds, biotransformation happens in lung tissue. After oral absorption, the liver is the main site for drug metabolism and can be involved in the first-pass elimination of a drug before it attains the systemic circulation. Special compound classes, e.g., peptides, predominantly undergo metabolism in the kidneys, other compounds such as many prodrugs are prone to metabolism by plasma esterases.

For the selection of the suitable in vitro test system, e.g., to investigate the metabolism in the liver, predictivity of in vitro–in vivo extrapolation ranges from whole liver perfusion studies to recombinant enzymes as shown in Fig. 1. In other words, decreasing complexity of a test system decreases their accuracy of prediction in the clinical state but allows rapid implementation and higher throughput. Selection of a particular test system depends on the required information at key decision points along the value chain of research and development (Dudda and Kuerzel 2006).

In Vivo Test Systems

Metabolism investigations in the whole body are key to obtain a complete picture of qualitative and quantitative biotransformation of a drug. Radiolabel studies are the method of choice in order to exhaustively detect and quantify all metabolites, including structurally unknown ones or those for which no reference compounds

are available (e.g., to allow quantification using standard bioanalytical assay methods). Moreover, use of radiolabeled compounds facilitates validation of sample processing for matrices like feces, organs, and tissue homogenates by determination of extraction yields or recoveries. The selection of animal species for these investigations is determined by the species used in chronic toxicology studies, both a rodent and a non-rodent species, in general rat and dog, as well as those additional species used in carcinogenicity (mouse) and embryofetal studies (rabbit). The human ADME study, investigating absorption, distribution, metabolism, and excretion in humans, is of particular importance to detect and quantify all human metabolites. The radiolabeled study in animals can help to define the human NOAEL (no-observed-adverse-effect level) dose and to help set a dose for human ADME study (Krone et al. 2011). Important matrices for analysis in the animal radiolabeled study are blood, plasma, urine, fecal homogenates, and in addition to animals' bile, milk, organs, and tissues (Fig. 2). Of importance is the difference in the metabolism due to sex, dose, race age, as well as possible persistent accumulation of the drug and/or its metabolites in organs and tissues as indicated in tissue distribution studies. Alternatively, to the classical human ADME study, a radiolabeled "microtracer" study could be applied with negligible amount of radioactivity, respectively of carbon-14. This study type allows the detection of drug related material with long internal exposure time, due to low systemic elimination of the drug and/or metabolites, or due to covalent binding to endogenous material. Though the sensitive detection of carbon-14 requires costly accelerated mass spectrometry (AMS) analytics, human metabolites can be monitored with this method already in the early clinical phase I studies (Frédéric et al. 2018).

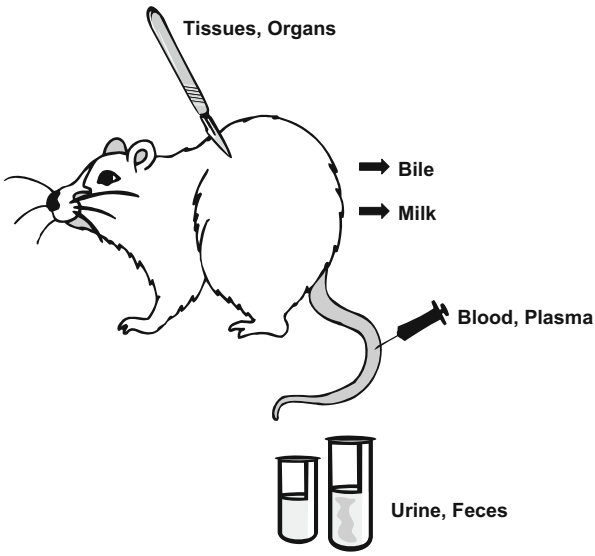

Fig. 2 Important matrices for in vivo metabolism studies

Structure Elucidation of Metabolites

Samples from in vitro studies, as well as radioactive ADME studies in animals and humans, and high dose nonradioactive in vivo studies are collected in order to elucidate metabolite structures by suitable techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, often coupled with liquid chromatographic methods ("LC-MS," "LC-NMR"), often after complex and time-consuming concentration and purification steps. The application of radiolabeled material enables the quantification and definitive detection of drug-related material. Pending on the radionuclide, on the amount and specificity of the radioactivity, and the demand of sensitivity, various detection methods (online with LC/offline in fraction collected/accelerated mass spectrometry) can be applied.

A definitive identification of the structure of relevant metabolites (abundant and/or pharmacologically active) is the prerequisite for their chemical synthesis. Availability of metabolites for further tests allows the bioanalytical quantification in all toxicological and clinical pharmacokinetic studies.

Regulatory Considerations on Metabolites in Safety Testing

For the safety assessment of a NCE and its metabolites recommendations are provided by Food and Drug Administration (FDA) (*Safety Testing of Drug Metabolites – Guidance for Industry, Revision 2, Final, March 2020*) and the European Medicines Agency (EMA) (*ICH guideline M3(R2) on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals, Revision 2 adopted guideline, December 2009*).

Generally, the safety testing of the metabolites is regarded as sufficient if the metabolic profile in humans is similar to that in at least one of the animal species used in nonclinical toxicological studies. But metabolic profiles "*can vary across species both quantitatively and qualitatively, and there are cases when clinically relevant metabolites have not been identified or adequately evaluated during nonclinical safety studies"* (FDA). The guidelines advice the early identification of metabolites "*found only in humans* (unique human) *and is absent in the animal test species, or if the metabolite is present at disproportionately higher levels in humans* (human disproportionate) *than in the animal species used in the standard toxicity testing with the parent drug"*(FDA). "*In vitro metabolic profile data for animals and human should be evaluated before initiating human clinical trials*" (EMA).

The focus is on metabolites formed from Phase I reactions (e.g., by oxidation, hydroxylation). These are more likely to be chemically reactive or pharmacologically active than Phase II conjugation products, with the exception of acyl-glucuronides. Both guidelines refer to a threshold of greater than 10% of total drug-related exposure at steady state of human metabolites that can raise a safety concern, and thus recommend the identification of the differences in metabolism as "*early as*

possible in the drug development" (FDA) or at least *"before exposing large numbers of human subjects or treating long duration"* (EMA).

For assessing metabolite safety, the FDA guideline provides detailed information on the different approaches for metabolite identification including recommendations of studies (general toxicity, genotoxicity, embryo-fetal development toxicity, carcinogenicity) needed to characterize the adverse potential of the disproportionate human metabolite.

Investigation of Drug–Drug Interactions

A drug is intended not only to be pharmacologically active with as little toxicity as possible but also as safe as possible in relation to both individual genetic differences of patients and concomitant administration of other drug(s). In the latter cases, only human in vitro metabolism systems are able to predict safety in human due to the lack of relevance of animals, due to significant enzymatic differences between animals and human. These in vitro experiments are the prerequisite to time-consuming, costly, and perhaps risky clinical studies. Various modelling approaches (e.g., physiologically based pharmacokinetic (PBPK) model) assist the translation of in vitro results into in vivo prediction, of potential in vivo drug–drug interactions (DDI), and provide the first assessment of the requirement to conduct a clinical interaction study (Fig. 3).

Elucidation of Enzymes Involved in the Metabolism of a Drug

Majority of drugs are eliminated from the body after biotransformation into more hydrophilic metabolites which facilitates their excretion. The safety or the efficacy of a drug in patients can be affected by the interindividual differences in enzyme activities or by co-medication, possibly leading to inhibition or induction of these enzymes. Thus, enzymes involved in the biotransformation of a drug have to be elucidated as early as possible in a phenotyping study. Of importance are enzymes revealing a genetic polymorphism. The major enzymes implicated in the biotransformation of most marketed drugs are the cytochrome P450s, a superfamily of enzymes and its many isoenzymes. Inhibition experiments in human liver microsomes can be performed using selective inhibitors or antibodies. In addition, human recombinant P450 enzymes are available to identify enzymes capable for metabolizing a drug. Correlation analysis of turnover observed in individual donors with different enzyme activities can be done. All these investigations contribute to verify or rule out the involvement of a particular enzyme. Similar studies can be applied to investigate non-P450 enzymes, although availability of recombinant enzymes or selective inhibitors might be limited.

The quantitative effect on the pharmacokinetics of the drug and a possible interaction with other co-medications has to be determined in clinical studies based on these in vitro findings.

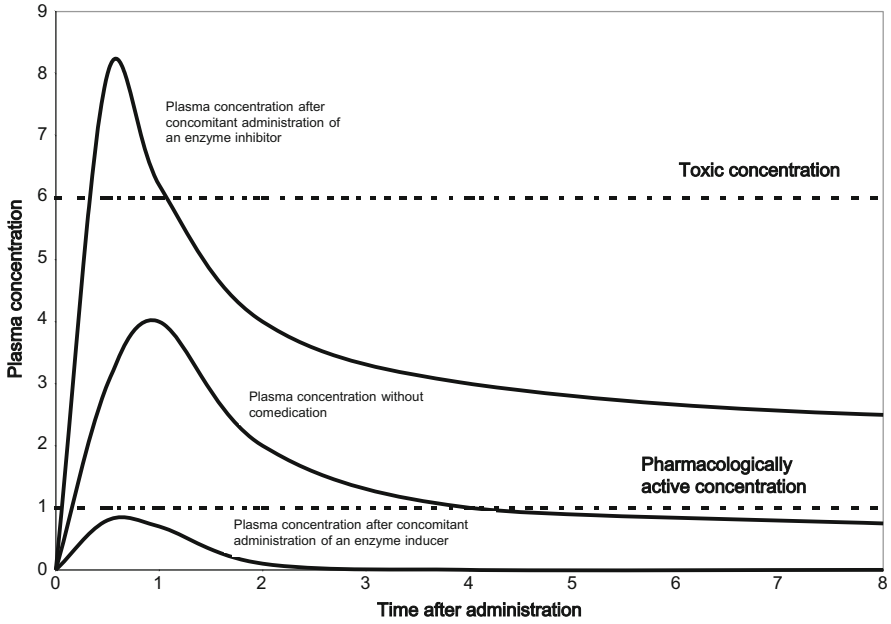

Fig. 3 Potential effect of co-medications on plasma levels of a drug by inhibition or induction of drug metabolizing enzymes

Enzyme Inhibition

A drug can act as an inhibitor of drug metabolizing enzymes through a reversible mechanism, e.g., competitive inhibition, or irreversible, e.g., covalent interaction. This can lead to a prolonged elimination of the victim drug and thus, to higher plasma concentrations, which are potentially toxic. The determination of the inhibition potential of a new drug is therefore important. This can be performed in human hepatocytes or liver microsomes applying enzyme-specific marker substrates or in recombinant human enzymes. Reversible inhibitors are characterized by determination of the IC_{50} or inhibition constant (Ki). Risk assessment based on these data is acknowledged by health authorities to or not to conduct a clinical interaction study.

Enzyme Induction

After repeated administration, drug metabolizing enzymes can be induced. The resulting increased enzyme activity can lead to a more extensive metabolism of a co-medication metabolized by this particular enzyme. Decreased plasma levels can lead to the loss of efficacy. Enzymatic induction can be evaluated at mRNA, protein, or enzyme activity levels and thus, can be studied by real-time PCR, western blot, or enzyme activity determination. Suitable in vitro test systems for the determination of

enzyme induction are human hepatocyte cultures using a 3-day incubation of the test drug. High-throughput screening in discovery is possible using reporter gene assays in cell lines, such as PXR.

Regulatory Considerations on DDI (Drug–Drug Interaction)

In January 2020, the US Food and Drug Administration (FDA published two final guidelines, one entitled "*In Vitro Drug Interaction Studies — Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions Guidance for Industry*" and the other entitled "*Clinical Drug Interaction Studies — Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions Guidance for Industry.*" They represent the current framework from the US health authority on how to conduct in vitro assays to evaluate the P450 and transporter mediated DDI potential, and how to conduct clinical DDI trials to assess the DDI risk in patients.

The in vitro FDA guidance recommends the evaluation of metabolism-mediated DDI of the drug and of its metabolite(s) (pending on pharmacological activity, exposure – expressed by area under the curve (AUC) – and polarity, compared to parent drug) before the first in human studies by the conduct of the following investigations:

1. Drug/metabolite is a substrate of metabolizing enzymes (phenotyping study) – for drug, if the enzyme is responsible for $\geq$25% of drug's elimination – for metabolite(s), if pharmacologically active and contributes to $\geq$50% of the overall activity
2. Drug/metabolite is an inhibitor of metabolizing enzymes (inhibition study) – for metabolite(s), if
   (a) The metabolite is less polar than parent drug, and $AUC_{Metabolite} \geq 25\%$ of AUC_{Parent}
   (b) The metabolite is more polar than parent drug, and $AUC_{Metabolite} \geq AUC_{Parent}$
3. Drug is an inducer of metabolizing enzymes (induction study).
4. Drug is a substrate or inhibitor of transporters (transporter study)

Detailed information on in vitro DDI test assays including a recommendation of the enzymes and transporters to be investigated, as well the models for the prediction of in vivo DDI are provided by the FDA guidelines, and are regularly updated.

The drug interactions guideline of European Medicines Agency (EMA) *(Guideline on the investigation of drug interactions, CPMP/EWP/560/95/Rev. 1 Corr. 2, June 2012)* gives a more general view of pharmacokinetic (PK) interaction of the NCE, considering the effect of food intake or of other medicinal products on the PK of the investigational drug, and the effect of the NCE on the PK of other medicinal products. A comparison of the recommendations of metabolism-mediated DDI the EMA guidelines shows a high similarity to those provided by the FDA.

Metabolism of Biotherapeutics

Interest of the field of biologics for new treatments is increasing. The number of approved therapeutic proteins is continuously increasing. These new biological entities (NBE) cover a wide range of proteins, including antibodies (IgG), antibody drug conjugates (ADC), nanobodies (VHH), and fusion proteins. These NBEs can undergo biotransformation, that usually does not include the typical phase I or phase II enzymes. The biotransformed protein may be pharmaceutically active, inactive, or, like in the case of antibody drug conjugates, potentially toxic (Schadt et al. 2019). A typical biotransformation reaction for therapeutic proteins is the proteolytic cleavage, leading to a truncated protein and peptides in the circulation. In addition to proteolytic cleavages, de-glycosylation, deamidation, oxidation, or other amino acid modifications can occur. The clearance of therapeutic proteins ultimately is associated with degradation to small peptides and individual amino acids (catabolism).

While for small-molecule drugs, strategies and approaches to assess biotransformation are well established (Schadt et al. 2018), and regulatory guidance for the assessment of metabolites is provided by the authorities (see section above), therapeutic proteins biotransformation studies are not conducted by default procedures, as the commonly expected consequence is the degradation to small peptides and individual amino acids. Therapeutic proteins are slowly cleared through proteolytic digestion mechanisms, partly after cellular uptake in, e.g., target cells, endothelial cells, and macrophages, with a typical half-life of approximately 3 weeks for conventional IgG antibodies. However, for complex multi-specific antibodies or fusion proteins, high unexpected clearance, with or without species differences as well as a mismatch in PK/PD relationship may trigger biotransformation studies, with the aim to understand observed differences in exposure and or in pharmacological activity. This reflects the agencies view that biotherapeutics may have a limited potential to form catabolites/degradation products with unwanted off-target activity. A guidance on preclinical studies is provided by the International Council for Harmonization (ICH) S6 (R1) "Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals" (ICH 2011).

Nevertheless, it can be important to optimize the candidate's stability to get favorable in vivo properties. Vulnerable positions in the molecule can be identified and be further reengineered, obtaining more stable constructs. The triggers for such biotransformation studies are commonly unexpected high clearance, species differences, mismatch in the PK/PD relationships, or observed differences between the results of bioanalytical methods (e.g., LC-MS assays vs. ligand bind assays).

The use of appropriate in vitro systems can facilitate the identification of vulnerable molecular positions with higher throughput by reduction of preclinical in vivo studies according to the principle of 3Rs (replacement, reduction, and refinement). However, simple systems such as plasma, serum, or subcellular fractions of tissues are poorly predictive, as a low biotransformation rate significantly limits such models, which do not contain the relevant proteases or/and cells concentration (Sender et al. 2016). Therefore, biotransformation studies are preferably conducted in animals. Serum and tissue samples are purified by using immunoaffinity methods followed by separation

of the biotherapeutics and their degradation products with high performance liquid chromatography (HPLC). Alternatively, two-dimensional HPLC can be used for sample preparation and separation, if the adequate immunoaffinity system is not available, or the metabolites are not retained by the system.

Then the analysis of biotransformation products can be achieved by mass spectrometry (MS). The most straightforward approach is intact mass analysis of proteins using high-resolution mass spectrometry (HRMS). It allows the measurement at high mass to charge ratio (m/z), the resolution of individual charge states, and the deconvolution to exact mass of proteins and their proteoform or biotransformation products. A complimentary approach to intact mass analysis is the "bottom up" analysis performed by the digestion of biotherapeutics, using site-specific proteases (trypsin). The lysine- and arginine-specific cleavage provides small charged peptides, which are easier to analyze and still retain information about molecular composition, and show subtle changes in the molecule in a more sensitive manner.

Quality Assurance

Health authorities do not require metabolism studies to be conducted under GLP (**G**ood **L**aboratory **P**ractice) or GCP (**G**ood **C**linical **P**ractice). However, due to the importance of these investigations for drug safety, it is recommended to conduct these studies in the spirit of GRP (**G**ood **R**esearch **P**ractice) to ensure quality and validity of the data. This requires study plans, standard operating procedures (SOP), proper documentation of analytical methods and results, but no need for Quality Audits.

Cross-References

- ▶ Biomolecules Versus Smaller Chemicals in Toxicology: ICH, EU, and US Recommendations
- ▶ Biopharmaceuticals: CMC Development "Points to Consider" from a Regulatory Perspective
- ▶ Importance of Xenobiotic Metabolism: Mechanistic Considerations Relevant for Regulation
- ▶ National and International Collaboration in Regulatory Toxicology
- ▶ Principles of Analytical Chemistry for Toxicology
- ▶ Toxicokinetic Models

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Toxicokinetic Tests

16

Jürgen Pauluhn

Contents

Abstract

Toxicokinetics is an integral component of toxicological studies in order to interrelate the administered dose with the associated effect. Pharmaco−/toxicokinetic tests are essential for judging absorption, bioavailability, and

J. Pauluhn (✉)
Formerly Experimental Toxicology, Bayer HealthCare, Wuppertal, Germany
e-mail: juergen.pauluhn@icloud.com

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bioefficacy across routes, regimens, and species. In case substances are administered by inhalation, it provides a means to identify substance accumulation and clearance in the lung. Also in other subdisciplines such as oral and skin toxicology, toxicokinetics is important for the assessment of persistence and bioaccumulation. Toxicokinetics includes analyses of temporal change of concentration profiles of the parent substance, its metabolites, or degradation products. Accordingly, toxicokinetic data provide indispensable estimates for systemic and organ-specific substance burdens and contribute significantly to the interpretation of toxicological data and their significance for humans. Many examples, mainly for inhalation testing, are provided.

Keywords

Absorption · Distribution · Biotransformation · Excretion · Pharmacokinetics · Inhalation toxicology · Lung

Objectives of Toxicokinetic Investigations

Toxicokinetics deals with the study and with the mathematical description of the time dependence from the disposition (absorption, distribution, biotransformation, and excretion) of xenobiotics in the whole organism. Absorption is the translocation of the administered substance to the blood stream. Once in the blood, the substance is distributed through the body and delivered to tissues, where it may leave the blood and enter the cells of the tissue or it may remain in the blood, particularly when bound to plasma proteins, and simply pass through the tissue. In certain tissues, such as the liver, the substance may be effectively removed from the body by metabolism. Other tissues, such as the kidney and lung, serve to eliminate xenobiotics from the body by excretion. The factors influencing the disposition are conceptualized in Fig. 1. Substances may show complex pattern distribution within an organism depending on the partition to and affinity of the particular tissue for the parent compound or its metabolite. When its absorption and distribution is complete, the concentration in blood depends on the amount absorbed and the extent of tissue distribution.

Knowledge of the specific time profiles of concentrations within tissues or specific compartments is important for assessing the total organ dose (AUC, area under the curve) as well the associated peak concentration (C_{max}). The AUC is represented best by the dose or concentration administered per unit of time × dosing duration ($C \times t$). Depending on whether any specific toxic outcome is AUC- or C_{max}-dependent, the same AUC can produce markedly stronger effects when C_{max}-dependent mechanisms play a role (Fig. 2). In case the rate of dosing exceeds that of elimination or clearance, a substance may accumulate at the portal of entry or the organ showing the highest partitioning/affinity to the substance administered. Typically, such accumulation occurs in the lung following long-term exposure to insoluble dust particles deposited and retained in the lower respiratory tract.

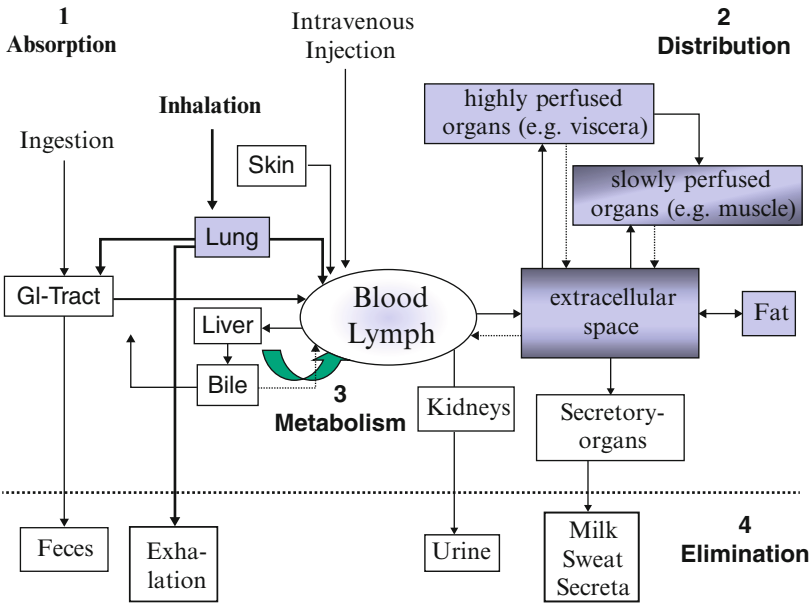

Fig. 1 The four steps of toxicokinetics: absorption, distribution, metabolism, and excretion (ADME)

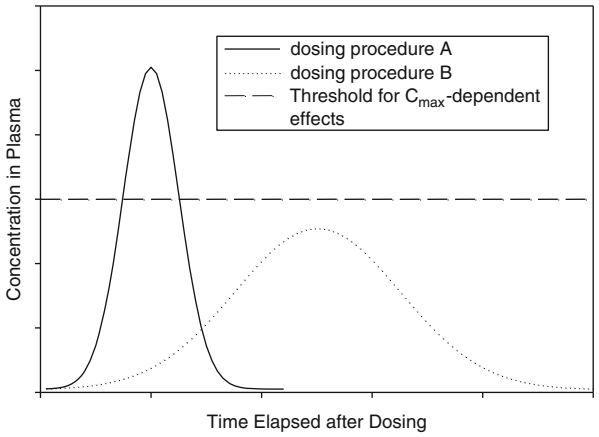

Fig. 2 Theoretical curves representing the plasma concentration of a chemical at different times of dosing. *Curve A* represents the systemic dose from a substance administered by gavage, which is rapidly absorbed. *Curve B* represents the systemic dose from the dermally applied substance that is slowly absorbed. Assuming the area under the two curves is the same and the elimination capacity becomes saturated at a plasma concentration shown by the *broken line*, the toxic effect in A will be greater than in B and extrapolation between the two routes will either underestimate (B to A) or overestimate (A to B) the hazard. C_{max}-dependent toxicity, such as neuroexcitation, can reliably be estimated by route A only

Cumulative doses lead to a compartmental (alveolar macrophages) lung overload that may overproportionally increase their residence time in the lung as detailed in the later sections.

For the inhalation route, as long as the dosing variables are kept constant, the well-known reciprocal relationship of concentration and time, that is, $C^n \times t = constant\ effect$, is a valid approach (Haber's rule is fulfilled with $n = 1$). This rule is commonly understood in inhalation toxicology as a constant product of the two factors "exposure concentration in the inspired air" (C) and the "duration" (t) during which this concentration is inhaled to yield an identical intensity of biological response. A third factor has been considered, namely, the actual volume inhaled by the animal during the exposure period (t). The negligence of this last variable implies that the inhaled volume of a specified concentration of a hazardous substance in air is constant across exposure groups and need not be considered any further. This simplification is subject to challenge, especially when analyzing $C^n \times t$ relationships of irritant gases in small laboratory rodents which are known to instantly change their ventilation and respiratory pattern concentration-dependently. Historically, Haber's rule has been used for time × concentration extrapolations assuming that each unit of damage is irreversible, that no repair takes place during the exposure period, and, therefore, that each unit of exposure is 100% cumulative.

Physiologically based pharmacokinetic (PBPK) models are being used increasingly by regulatory agencies to estimate the internal dose of toxic/drug agents or their metabolites to target tissues. Using this technique, risk assessment for toxic substances can be based on estimates of the amount of the agent that reaches the target tissue, rather than the applied dose. In PBPK modeling, the pharmacokinetic behavior of a compound in the various compartments of the body is represented by equations that attempt to quantitatively describe actual physiological processes. The parameters of these equations are key anatomical and physiological descriptors of the organism. Thus, compartmental pharmacokinetic modeling is a powerful in silico tool for interrelating the accumulated target organ dose with the associated toxicological effect. Such models are useful for designing organ-specific drugs, "equivalent dose" testing protocols across species at differing exposure regimens, and to verify route-to-route extrapolations. Toxicokinetic studies pursue multiple objectives; these are summarized in Table 1.

Extrapolation Across Species and Systems

Testing guidelines recommend standardized approaches on selected animal species for the evaluation of specific toxicological endpoints. The use of common laboratory animals for inhalation toxicity studies continually supplements the database and furthers the understanding of toxicity data in experimental animals and their relevance for man. Kinetic cornerstones become ultimately important when examining novel substances with a species-specific mode of action. When using alternative species or testing approaches, kinetic data from target organs are indispensable to

Table 1 Requirements for kinetic analyses

Data requirement	Test system	Finding
Disposition "kinetic base set"	Single-dose studies to evaluate route-specific kinetic cornerstones	C_{max}, t_{max}, AUC, $t_{1/2}$ bioavailability, absorption rate (flux), parameterization for PBPK modeling, and invasion and evasion kinetics
Disposition, "accumulation," and organ-specific toxicity	Repeated dose study (e.g., oral, dermal, inhalation, intranasal, and intravenous)	Parameterization for PBPK modeling, distribution within the organism at steady state, organ burden vs. associated organ-specific toxicity, saturation and/or adaptation, and accumulation
Dosimetry and biomonitoring	Single to repeated administration/exposure (e.g., oral, dermal, and inhalation)	Proportionality of external or administered dose with "internal" biomarkers of exposure, including fate. Each exposure pathway displays its own relationship
Across-species comparisons/ extrapolations	Single to repeated administration/exposure (e.g., oral, dermal, inhalation, intranasal, and intravenous)	Species-specific differences in disposition, protein binding, organ burden, and associated toxic effects
Route-to-route extrapolation, exposure regimens	Single to repeated administration/exposure (e.g., oral, dermal, and inhalation)	C_{max} and AUC of the parent chemical or metabolite in the blood or selected tissues
Modulation of absorption overload	Single to repeated administration/exposure (e.g., oral, dermal, and inhalation)	Vehicle, particle size, including solubility, surface area, and surface functionalization; amorphous or crystalline; excipients to modify absorption/clearance for drug delivery applications; competitive effects
PBPK-based study design	Single to repeated administration/exposure (e.g., inhalation)	Optimization of particle size for pulmonary deposition, dose selection based on TK properties (accumulation, dissolution, and translocation) of particles to attain lung burdens at nonoverload to overload

disentangle effects in regard to their dynamic (susceptibility) or kinetic (disposition) cause. The choice of animal species for regulatory testing is usually based on guideline requirements and practical considerations rather than validity for use in human beings. An animal species must be small enough to allow handling and exposure in sufficient numbers in relatively small inhalation chambers. An animal species, however, must be large enough to allow measurement of all endpoints relevant to identifying the inherent toxicity of the substance under investigation. Exposure paradigms may vary from small to larger animals, from animal bioassays to humans, as well as within the human population.

In summary, no animal species mimics man in all respects. Therefore, animal models are, at best, a necessary compromise and must be used because they offer the advantages of experimental control and reproducibility. Accordingly, animal model selection may often be contingent upon toxicodynamic or pathological identification of early changes consistent with the pathomechanism of the test substance. In general, the uncertainties of extrapolation of toxicological results across different species are minimized if a maximum of mechanistic understanding is gained from a study. This is achieved by measurement of a sufficient number of procedures, endpoints, and incorporation of kinetic endpoints accounting for any species-specific differences in dosimetry and fate.

Recent progress in in vitro toxicology makes it necessary to consider pharmacokinetics also on cellular level to make comparisons possible of in vivo and in vitro models. New methods in the cultivation and exposure allow the direct exposure of lung cells thus providing means to analyze biological responses of cells during and following direct exposure to airborne materials at the air/liquid interface. Such systems are amenable of using cultured cells as an integrating biological dosimeter. Nonetheless, one has to recall that in vitro systems are typically designed for homogenous systems with water-soluble substances rather than insoluble and lipophilic particles which may have limited access to cells in aqueous culture media. To make cellular in vitro systems more practicable to human risk assessment, improved concepts for cellular dosimetry and kinetics are urgently needed.

Extrapolation Across Routes

Risk assessors frequently have to use data obtained using a single route of administration. Most studies are carried out using the oral route (by gavage or in diet or drinking water). This is because such studies tend to be the most straightforward to perform and interpret, and dosimetry is easiest to quantify, particularly when a chemical is given by gavage. However, the differences that may occur due to exposure via high "bolus" systemic dosing resulting from administration by gavage, intratracheal or intranasal instillation, compared with prolonged systemic dosing resulting from administration via food or drinking water, the skin, or inhalation, need to be recognized. If the metabolism or action at the target site of a chemical is critically influenced by concentration at any one time, rather than the total integrated systemic dose, the toxicity of that chemical may differ with route of administration or dosage regime if these affect the systemic concentration $\times$ time relationship (AUC) as shown schematically in Fig. 2.

The toxicity of a chemical may also be altered by previous exposure, for example, as a result of induction of metabolizing enzymes. Although these effects of the dosing regimen are important factors in the assessment of hazard, they are not specific to route-to-route extrapolation. Inhalation toxicity studies are technically much more complex: It is necessary to monitor the levels to which the animals are exposed, and in the case of aerosols, their size matters as it will influence the amounts that penetrate the upper respiratory tract (inhalable) and the alveoli

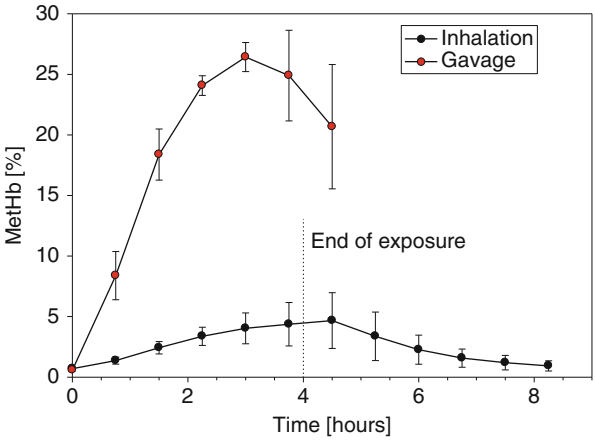

Fig. 3 Time course of methemoglobin (MetHb) formation in beagle dogs. The dogs were either head-only exposed to aniline vapor for 4 h to 174 mg/m^3 or received 15 mg aniline/kg body weight by gavage. Blood was collected before exposure and thereafter in 45 min intervals. (Data reproduced from Pauluhn (2002))

(respirable). Other factors that govern deposition include water solubility and reactivity. Calculation of dose is much more complex compared with using the oral route, being dependent on the lung morphology, ventilation, etc., as well as the applied C × t. There is therefore commonly a need to extrapolate toxicity data obtained using the oral route of administration in order to assess human health risks resulting from inhalation exposure.

Toxicity via oral and inhalation routes can differ remarkably when a dose rate-specific-activating first-pass metabolism occurs. Aniline is given as an example. This substance is known to be bioactivated in the gastrointestinal mucosa and especially in the liver to become a MetHb-forming agent. It exerts toxicity to the red blood cells (RBC) through an active/reactive metabolite mechanism, mainly N-hydroxylated metabolites, which take part in cyclic redox processes. This *vicious cycle* depletes the RBC of factors to regenerate oxidized hemoglobin. Dogs were exposed by head-only inhalation to exclude dermal uptake for 4 h to 0.174 mg aniline/L air. When applying the typical respiratory minute volume of dogs, the total exposure dose is equal to 15 mg/kg body weight (0.36 L/kg-min × 240 min × 0.174 mg/L = 15 mg/kg). The same dose was administered by gavage (Fig. 3). A fivefold lower potency of MetHb formation was observed following inhalation as compared to the bolus gavage. This appears to be related to the more efficacious hepatic first-pass bioactivation when administered via the gastrointestinal tract. Thus, for agents known to be bioactivated by a hepatic first-pass metabolism, the conversion of findings obtained from oral dosing to inhalation exposure concentrations is subject to errors. Likewise, in contrast to studies where the uptake is by the gastrointestinal route, the passage time-dependent modification of the test agent may be decisive for the toxic outcome.

Likewise, complex molecules can spontaneously decompose pH-dependently in the gastrointestinal (GI) tract as shown for the zinc-propylenbisdithiocarbamate fungicide propineb (Fig. 4). The degree of decomposition can be estimated by the nonenzymatically formed reaction product TTCA (2-thiazolidinethione-

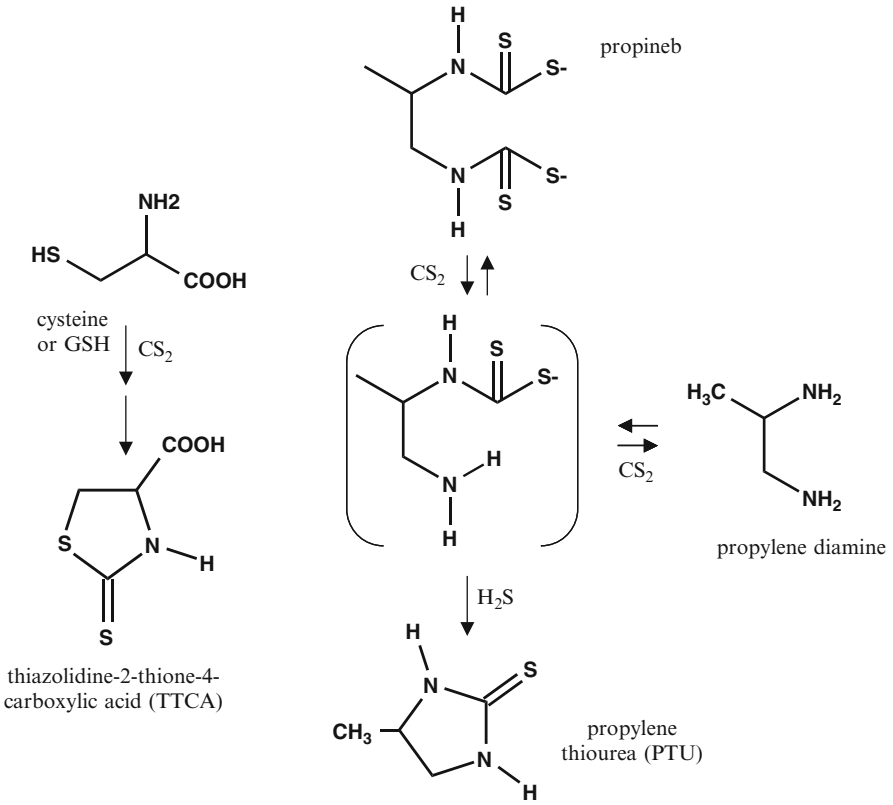

Fig. 4 Conceptual scheme of decomposition of the zinc-propylenbisdithiocarbamate fungicide propineb under physiological conditions showing the generation of carbon disulfide (CS_2) and the subsequent reaction with free cysteine (or the cysteinyl moiety of glutathione) to form a metabolite that cyclizes to TTCA. In concept, this decomposition may also lead to the formation of propylene thiourea (PTU), a thyreotropic substance. (Adapted from Pauluhn and Rosenbruch (2003))

4-carboxylic acid) in urine, a metabolite and biomarker of exposure to CS_2, through direct reaction with cysteine or glutathione. The dithiocarbamate formation is reversible under physiological conditions and provides a reservoir of CS_2 within biological systems. As can be deduced from Fig. 4, the intermediate concentrations of CS_2 and TTCA, including the new toxic entity PTU (propylenthiourea), and ionized zinc are substantially different following gastrointestinal and pulmonary exposure. Portal-of-entry-specific pulmonary changes may occur at lower doses due to the higher local concentrations of zinc and a diminished capacity of metabolizing CS_2. This makes different patterns of distribution likely to occur. In contrast to dietary routes, thyroidal (goitrogenic) effects have not been found following inhalation exposure, presumably because PTU is unlikely to be formed within the pulmonary environment. Thus, the formation of new toxicophoretic entities and portal-of-entry specific types of toxicities, including site-specific, often

rate-dependent compensatory mechanisms, needs to be accounted for before attempting such extrapolations.

The above considerations suggest that extrapolations from the oral to inhalation routes are by far not straightforward in the absence of adequate toxicity and biokinetic data. For route-to-route extrapolation to be appropriate, the toxicological concern often relates to systemic toxicity, that is, toxicity expressed in tissues/organs distant from the site of administration. However, even at this level of simplification, expert judgment and PBPK data are needed to arrive at scientifically sound conclusions.

The key factors that need to be considered are briefly summarized below:

- Is the toxicity of concern a local or systemic effect?
- Is there any dependence on the extent of absorption and on the balance between any activation (where appropriate) and detoxification mechanisms? In case this can be materialized, the systemic toxicity may be the result of complex interactions, some of which could be route specific as well as dose-rate specific.
- Absorption is a determinant as to how efficiently a substance can be transported across biological barriers of the target organ dose, and implications of first-pass-like metabolism.
- Decomposition of a substance within the gastrointestinal tract with the formation of new toxicological entities (toxicophoresis) needs to be appreciated. This means the toxicodynamics and spectrum of organ toxicity change from one route to another.

Toxicokinetic Data and Risk Assessment

In the application of pharmacokinetic models in risk assessment, the differences in compound disposition between humans and experimental animals are of particular interest. Therefore, there is a need to establish appropriate physiological values for humans and for the more commonly used laboratory species. To address this need, representative values and biologically plausible ranges of these values are provided for a number of anatomical and physiological parameters in multiple species. Representative values are available from the literature for most of the species commonly used in toxicology and risk assessment. As a result, these values can serve as reasonable, empirically based defaults that can be used in PBPK modeling when case-specific data are unavailable. Clearly, it is preferable to determine physiological and anatomical values directly on the individuals under study or, at least, on individuals known to be drawn from the same population and subjected to similar conditions.

Toxicokinetic studies provide historically an integral part of toxicological studies with drugs in order to allow direct comparisons of internal doses from preclinical animal studies to humans dosed with optimized drug delivery systems under clinical conditions. They also allow a specific search for the most relevant human-like animal species in regard to metabolism and pharmacodynamics.

Toxicokinetic Readouts

Time-Course Changes

Toxicokinetics commonly captures concentration measurements in plasma or blood of the parent substance or its metabolites. In inhalation toxicology, the information on extrapulmonary organ burdens, including blood, and lung burdens is of paramount importance for inhalation dosimetry and risk assessment. Apart from non-inhalation routes, physical factors may determine whether inhaled substances are cleared from the lung into the GI-tract with or without absorption. They also play a role in the lung where their kinetic of dissolution in pulmonary surfactant differs appreciably from that after their phagocytosis by alveolar macrophages. The elimination kinetics of insoluble particles from the lung depends on the degree of lung overload (see below). Thus, kinetic hallmarks characterizing the threshold dose of lung overload are a basic requirement for any meaningful extrapolation from animals to humans. Commonly, toxicokinetics focuses on the following parameters, AUC, which is a measure of the total systemic exposure to the chemical. AUC is an integral of the rate of change of concentration in plasma as a function of time. C_{max} is the maximum plasma concentration (see Fig. 2), and t_{max} is the time required to reach this maximum concentration after administration. They are determined at various time points after a dosing. Bioavailability, which is the fraction of chemical that is absorbed, is determined for extravascular administration with reference to an intravenous dose. An example for the compartmental kinetics of poorly soluble inhaled particles from the lung is given below (see Fig. 5).

Selection of Compartment

The pharmacological efficacy of a drug substance depends on its time-dependent concentration at the site of targeted action (target organ). Especially in humans, this target site is often not directly accessible for specimen collection. As a surrogate, concentration of drug is commonly determined in the blood assuming a state of equilibrium among organs. Drugs targeted to the lung are preferentially administered by inhalation to increase the C_{max} and AUC within this organ at equally lower systemic C_{max} and AUC which then substantially reduce the systemic dose with less side effects. As a result of this strategy, the concentrations in the blood and in the target tissue may differ considerably. PBPK models can be used to calculate the most appropriate dosing interval to prevent accumulation and local toxicity from occurring.

Aminoglycosides have several potential antibiotic mechanisms and are frequently administered by inhalation to attenuate/cure pulmonary infections. This class of drugs is known to have strong, irreversible binding to the ribosome and remains intracellular long after plasma levels drop. This allows a prolonged dosage interval. Depending on their local concentration, they act as bacteriostatic or bactericidal agents. These properties make these drugs amenable to interact inhaled dose proportionally with the phospholipids lining the lung resulting in dysfunctional

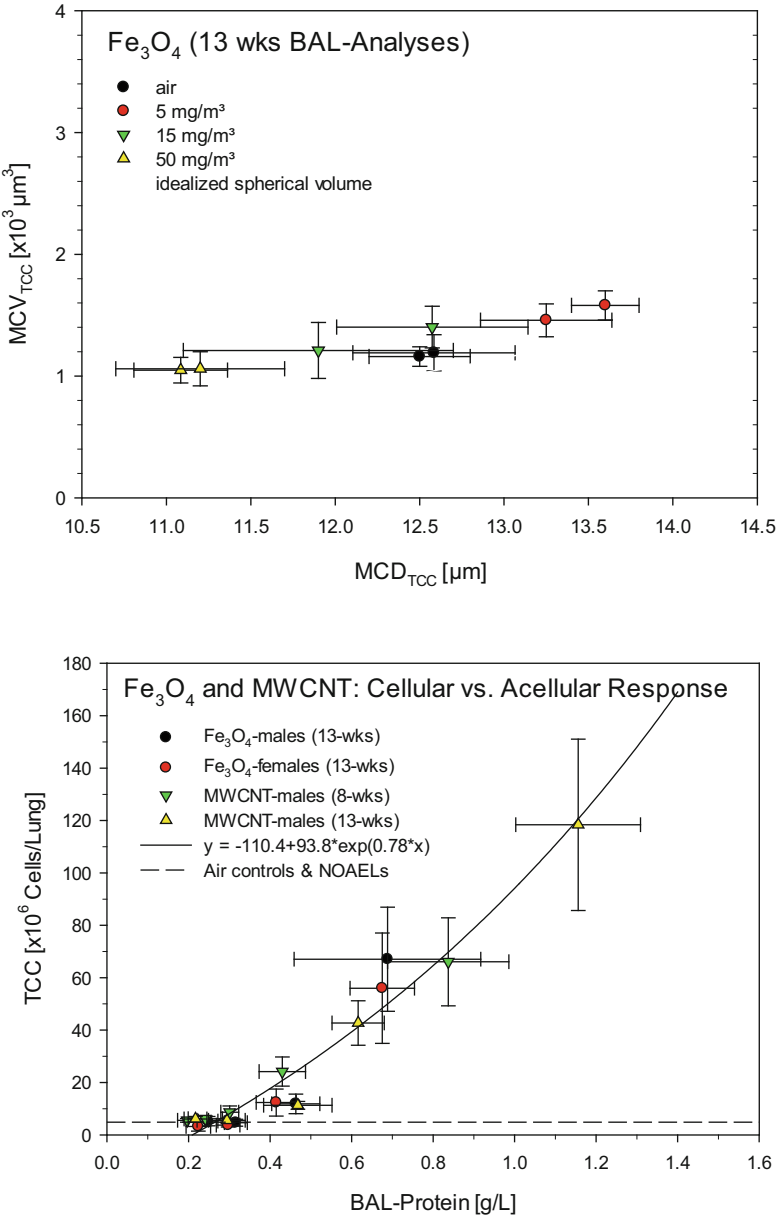

Fig. 5 **Top** Comparison of the mean corpuscular volumes (MCV) and diameters (MCD) of total cell counts (TCC) in the BAL of rats exposed to Fe_3O_4 as modeled in Fig. 6-top. **Bottom:** Interdependence of BAL-TCC and BAL-protein (Fe_3O_4: male and female rats, 13-wks sacrifice; MWCNT: male rats, 8 and 13 wks sacrifices). Animals were exposed to MWCNT and Fe_3O_4 as illustrated in Fig. 6-top. Whiskers represent the mean ± SD of six male rats/group examined at the end of study. This comparison demonsrates that the size and volume of the phagocyte (alveolar macrophage) is not appreciably affected by lung overload. However, the pool size of phagocytes (TCC) parallels favorably the degree of lung overload. Notably, the low density nano-MWCNT and high density iron oxide follow the same principle of volumetric lung overload

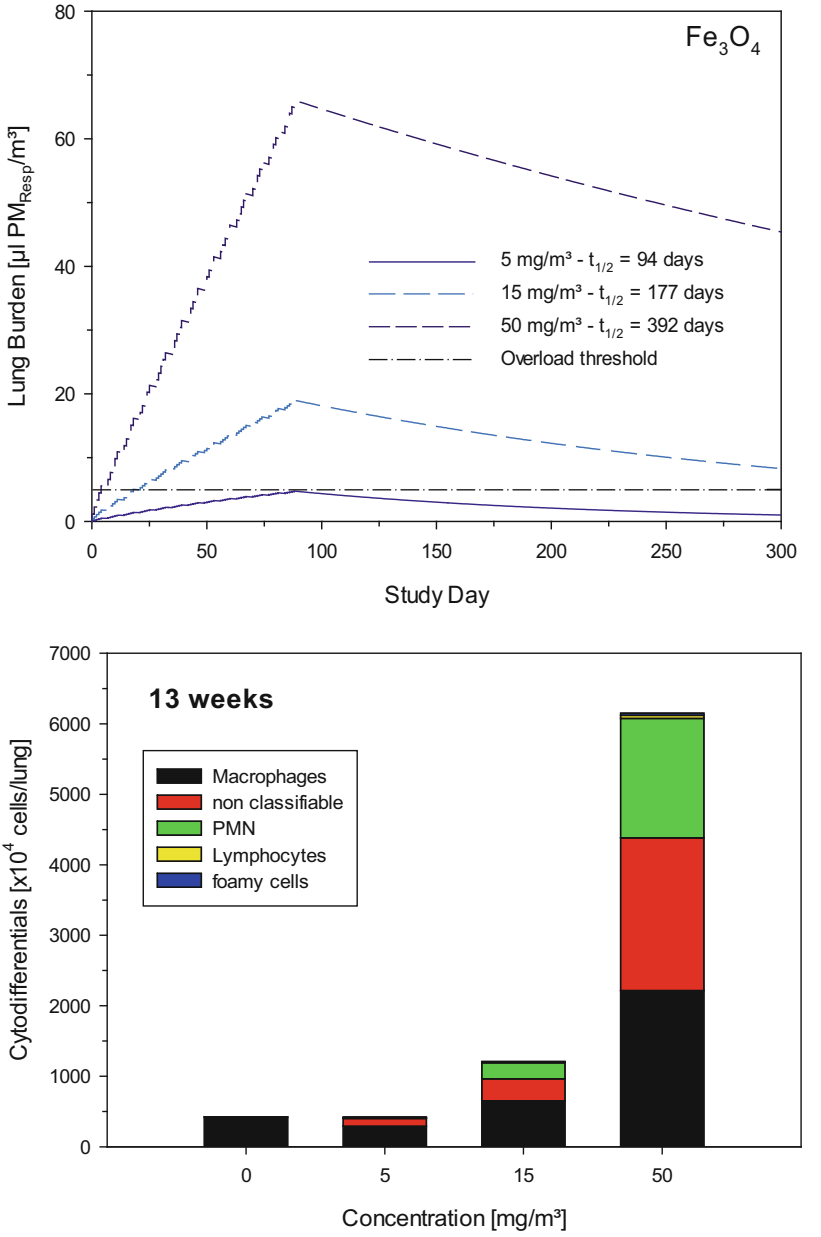

Fig. 6 Modeling of time- and particle volume-dependence of cumulative lung burdens of Fe_3O_4 solid aerosol in rats exposed for 13 weeks for 6 h/day on 5 days/week. The exposure concentrations selected were modeled to be tolerated without effects at 5 mg/m^3 and mild-to-moderate reversible overload-dependent pulmonary inflammation at 15 mg/m^3. Kinetic overload occurred at 50 mg/m^3, respectively (top). The cytodifferentiation of BAL-cells (end of exposure period) mirrors the modeled time-cumulative inhaled dose-response relationship (bottom). Nonclassifiable cells are macrophages stained black as a result of the engulfment of black magnetite

surfactant due to aminoglycoside-phospholipid complexes which are engulfed by alveolar macrophages. Dose-proportional elevations suggestive of drug accumulation in the lung provide evidence of reversible drug sequestration in the lung with clearance proportional to the degradation of drug-phospholipoprotein complexes. By virtue of PBPK modeling, the complex relationship of drug sequestration and elimination can be estimated by PBPK modeling in spite of its potential to accumulate.

Absorption, Distribution, and Elimination

Absorption

For drugs, the absorption is usually as large as technically feasible to maximize systemic bioavailability or alternatively small when limited to the site of application (e.g., the lung). Absorption can potentially take place from all exposure routes (oral, dermal, inhalation, and intranasal). The absorption rates and yields are highly route- and excipient-dependent or formulation-dependent. Therefore, PBPK modeling requires exposure information to account for these differences. Biomonitoring using biomarkers of short-term or long-term exposure integrates the dose from all exposure routes. Caution is therefore advised to attribute data to one single route without considering the potential impact of other routes.

For rapidly absorbed chemicals, equilibrium may be established between blood and the site of absorption. The rate of entry into the blood is limited by the blood flow rather than any diffusion across the membrane barrier. In this case, any increase in blood flow will also increase the rate of absorption, and absorption is said to be perfusion- or blood-flow-limited. For poorly absorbed substances, absorption is not dependent on the blood flow which means it is diffusion-rate-limited. Absorption is taking place by passive (mainly via pores or simple diffusion processes), active transport (against a concentration or electrochemical gradient) utilizing transcellular (through the cell) or paracellular (between cells) pathways. Solubility is an important factor driving the rate of absorption. For instance, particles deposited in the lower respiratory tract, in the absence of any specific protein binding or chelation, even less soluble substances (solubility in the range $\leq$ 1 mg/L water at physiological pH) are still rapidly cleared from the lung by dissolution.

Absorption from the Skin

For the penetration and absorption of substances through the skin, the flux needs to be calculated using Fick's first law of diffusion to relate the flux rate (J in mg/cm^2/h) to the permeability, concentration, area of exposed surface, and length of exposure. An additional important parameter that needs to be utilized is the chemical's permeability coefficient (Kp). The permeability coefficient (in cm ^{2}/h) should be

consistent regardless of exposure concentration (provided that the concentration is infinite) and surface area for any given exposure site and chemical, but it can vary between exposure sites. Unless a mathematical model is used, the calculation of flux or permeability coefficient must be assessed at steady state. Mathematical models have been developed to attempt to describe percutaneous absorption kinetics. In almost every case, absorption through rodent skin is more than threefold higher than through human skin, and the increased absorption through rodent skin does not show a consistent pattern between compounds. The higher absorption in rodent skin may be due to differences in skin appendages (e.g., hair follicles) and different morphology of the individual skin layers. An additional difference between human and rodent dermal absorption is the difference in the lag phase before the appearance of chemical in the blood; human absorption is delayed, whereas chemical absorption through the skin of rodents occurs with no apparent delay.

Gastrointestinal Absorption

The rate of gastrointestinal absorption from the gastrointestinal tract depends on the pK_a, solubility, lipophilicity, and degree of ionization, as well as its residence time which is influenced by the tract's filling state, pH of microenvironment, and the active surface area of the respective segment. The milieu of the GI tract and the respective bacterial microflora can promote spontaneous intraluminal decomposition or chemical modification of substances (e.g., formation of nitrosamines in the stomach). Metabolic activation or deactivation of the absorbed substance from the intestine can take place in the subsequent passage through the liver (first-pass metabolism). The rate and extent of absorption of weak organic acids and bases varies with the location in the GI tract; weak acids are nonionized and are absorbed in the stomach, whereas weak bases are nonionized and are absorbed in the intestine. Removal from the site of absorption by blood flow maintains a concentration gradient, thus enhancing absorption of chemicals. The protonation of lipophilic chemicals within any specific subcompartment may lead to intracellular substance accumulation by a "pH trap."

Pulmonary Deposition, Retention, Clearance, and Absorption

The various species used in inhalation toxicology studies do not receive identical doses in comparable respiratory tract regions when exposed to the same external particle or gas concentration. The total body burden per unit body weight may also differ from one species to another because of differences in respiratory patterns and the respiratory minute volume. The dose metrics is also dependent of the local and/or systemic pathomechanism and may range from "total body burden" to "critical dose per alveolar macrophage" or "critical dose per cell

volume or surface area of the most susceptible lung region." The biologic endpoint or health effect of concern may be more directly related to the quantitative pattern of mass deposited within the respiratory tract than to the external exposure concentration.

Retention is the actual amount of inhaled agent found in the lungs at any time and is determined by the relative rates of deposition and clearance. Retention and the toxicologic properties of the inhaled agent are related to the magnitude of the pharmacologic, physiologic, or pathologic response. For particles, deposition mechanisms include inertial impaction, sedimentation (gravitational), diffusion, interception, and electrostatic precipitation. Generalizations regarding the site of deposition of particles of a given size are problematic due to the many factors involved. However, in the average adult human, most particles larger than 10μm in aerodynamic diameter are deposited in the nose or oral pharynx and are unlikely to penetrate to tissues distal to the larynx. Very fine particles (0.01μm and smaller) are also trapped relatively efficiently in the upper airways by diffusion. Particles that penetrate beyond the upper airways are available to be deposited in the bronchial region and the deeper-lying airways. Sedimentation brings about deposition in the smaller bronchi, the bronchioles, and the alveolar spaces, where the airways are small and the velocity of airflow is low. As a particle moves downward through air, buoyancy and the resistance of air act on the particle in an upward direction, while gravitational force acts on the particle in a downward direction. Eventually, the gravitational force equilibrates with the sum of the buoyancy and the air resistance, and the particle continues to settle with a constant velocity known as the terminal settling velocity. Diffusion is an important factor in the deposition of submicrometer particles or gases. The clearance of deposited particles is an important aspect of lung defense. Rapid removal lessens the time available to cause direct tissue damage. Particles are cleared by the mucociliary escalator from the airways or may be phagocytized by alveolar macrophages and are ultimately transported to the mucociliary escalator. Even moderately soluble particles dissolve relatively rapidly in the lining fluids of the lung.

The mechanisms important for gases include convection, diffusion, chemical reaction (including metabolism), dissolution, and perfusion. Especially in obligate nose-breathing animals, absorption or "scrubbing" of a relatively water-soluble and/or reactive gas may occur from the inspired airstream as it travels from the extrathoracic to the pulmonary region. That is, the dose to the peripheral regions is affected by the dose to the region immediately proximal. For lung irritants, commonly an anterior-posterior gradient of intensity of damage of airways is observed, whereas the severity of toxicity also progresses distally with increased exposure concentrations. Although the deposition, clearance mechanisms, and physicochemical properties of the agent are often described as distinct properties, assessment of the overall toxicity requires integration of the various factors. Regional deposition pattern determines not only the initial lung tissue doses but also the specific pathways and rates by which the inhaled agents are cleared and redistributed or translocated.

Compartmental Pulmonary Biokinetics of Poorly Soluble Particles

Kinetic Lung Overload

The elimination of deposited and retained poorly soluble particles (PSP) from the lung usually is a logarithmic process – that is, a constant proportion of the substance is eliminated per unit time which is described by a first-order relationship: $C_t = C_0 e^{-kt}$ where C_t is the concentration after the time t, C_0 is the initial concentration at $t = 0$, and k is the elimination constant (k_e). The relationship between the elimination rate constant (k_e) and halftime ($t_{1/2}$) is given by the following Eq. 1:

$$C = C_0 e^{-k_e t} \text{with } k_e = \frac{\ln 2}{t_{1/2}} \tag{1}$$

Halftime is dependent on the clearance (CL) and the volume of distribution (V_d) which are combined using the following relationships:

$$t_{1/2} = \frac{\ln 2 \times V_d}{CL} \tag{2}$$

with

$$CL = V_d \times k_e \tag{3}$$

There is a common strong relationship between the volume of distribution (Vd) and body weight across species. This aspect has been observed when adjusting this endpoint across species. Regarding the lung, the Vd increase with the increased particle retention and load by alveolar macrophages (AM). The increased Vd decreases the clearance of PSP from the lung. In other words, the *greater the* Vd, *the longer* is the elimination halftime. This has significant bearings on the elimination kinetics of PSP being sequestered within a dynamically increasing pool of the migratory AM. The size of this Vd-equivalent pool is determined by the cumulative retained displacement volume within the AM followed by an adaptive influx of alveolar macrophages. The displacement volume is the space consumed by agglomerated PSP within the AM. This explains why low-density PSP commonly appear to be more toxic as compared to high-density materials because a smaller mass displaces a larger volume within the AM. However, this apparently higher toxicity is caused by an earlier attainment to kinetic lung overload and not necessarily any substance-specific toxicity. Once endocytosed by the AM, PSP may undergo multiple processes such as dissolution and/or elimination by translocation of particle-laden AMs out of the pulmonary system. Notably, dissolution is by far not trivial because dissolution may be facilitated or impeded depending on the localized pH and binding capicity of the adjacent microenvironment and its degree of saturation, When escaping this microenvironment, metal cations may be scavenged by chelating proteins or undergo precipitation often of much lower solubility than the parent PSP.

Thus, unlike the substance-specific kinetic cornerstones of soluble materials, the kinetic cornerstones of PSP are determined by the degree of the cumulated particle

load, called "kinetic lung overload." Accordingly, with increasing lung particle burdens, the increased elimination halftime is causally linked to the pool size of phagocytes and not any significantly increased size of the AM (Fig. 5, top). This illustration delineates that the average diameter of cells retrieved by bronchoalveolar lavage (BAL) yields a cellular diameter of 12.5μm in air-exposed control rats with a ± 10% shift into both directions. BAL cytodifferentiation from control rats is predominated by AM whereas that of highly dose rats contains also neutrophils. Overall, the relationship shown in Fig. 5 (top and bottom) demonstrates clearly that the pool size of AMs is determined by the increased number of BAL-cells constituting Vd rather than any significant change in cellular volume. The relationship depicted in Fig. 5 (bottom) compares iron oxide (Magnetite, Fe_3O_4; concentration: 5, 15, and 50 mg/m^3) and Multiwalled Carbon Nanotubes (MWCNT; concentrations: 0.1, 0.4, 1.5, and 6 mg/m^3) with specific densities of 4.5 and 0.2 g/mL powder, respectively, and exemplifies the dependence on particle volume, increased pool size of total cell counts (TCC), and ensuing pulmonary inflammation evidenced by increased extravasated BAL-protein.

The clearance of deposited particles via endocytosis is an important aspect of the constitutive lung defense. Rapid removal lessens the time available to cause direct tissue interaction and damage. Retention is defined as the actual mass of inhaled particles found in the lungs at any postexposure time and is determined by the relative rates of deposition and clearance. Those PSP deposited in the alveoli are primarily phagocytized by AM which are ultimately transported toward the mucociliary escalator with mechanical clearance via the airways. A small fraction of particles is also cleared via the lymphatic (interstitial) draining system draining the lung. The translocation of particles to the draining hilar lymph nodes (LALNs) commonly increases significantly at lung burdens high enough to cause pulmonary inflammation with increased barrier disruption and access to the interstitial system. PSP may also be cleared via the pleura, depending on their morphology (granular vs. fibrous). The pleural region of the parietal pleura was identified as a critical region rich in which all particles and fibers that are small enough to negotiate the parietal pores (stomata) are cleared, if not, they may accumulate at this mesothelial location followed by site-of-accumulation specific adversities. In rats, particles are preferentially retained in the airspaces, whereas in humans chronically inhaled PSP gain increasingly access to the interstitium.

As a corollary of their function, AM engulf and retain inhaled particles. As illustrated in Fig. 5, with increasing volumetric lung burdens, this may lead to an increased recruitment of phagocytic cells constituting the Vd. The relationship of accumulated lung burden and resultant terminal outcomes suggestive of lung overload-related inflammation at 13-wks are modeled in Fig. 6 (top). The PBPK modeling of lung burdens demonstrates that no-adverse-effect levels can reliably be predicted. Notably, under conditions of lung overload, lung burdens (and associated toxic effects) increase overproportionally due to the fractionally increasing elimination halftime as a result of kinetic lung overload. Toxicologists must observe this mismatch of clearly defined and spaced exposure concentrations relative to the overproportionally increased lung burdens at the end of repeated exposure studies.

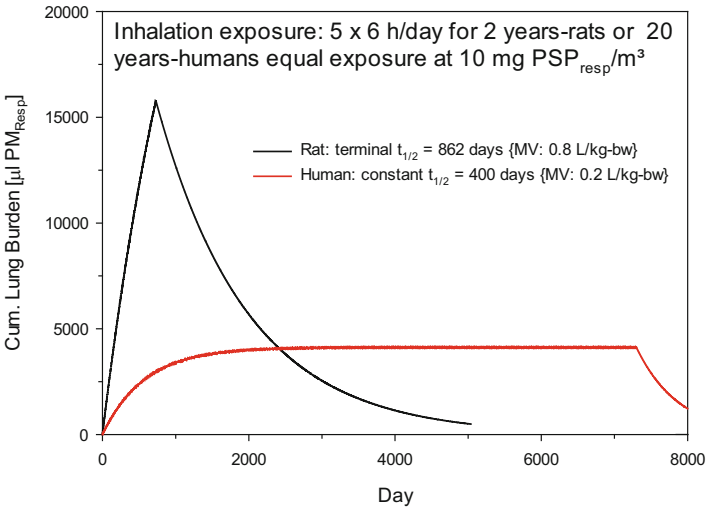

Fig. 7 Simulation of retained respirable volumes of PSP in rats and humans using a 5-days-per-week exposure (6 h per exposure day), over a prolonged period. Overproportionally, lung burdens may occur in rats under the conditions of lung kinetic lung overload whereas the larger Vd of humans prevents such state of overload to occur. Caution is advised to compare nominally inhaled cumulative lung burdens across species by "Area Under the Curves" in the absence of kinetic profiles

Regarding the available pool size for inhaled PSP, differences between laboratory rats and humans must be observed. Humans have large and higher numbers of AMs compared to rats. Hence, the fractional exhaustion of pool size changing an entirely adaptive process toward a potentially adverse process needs to be conceptualized. Accordingly, when exposed to the same concentration of PSP, rats may produce overload-specific adversities whereas humans still maintain in the state of non-overload (Fig. 7).

Thus, unlike humans, rats appear to be more susceptible to overload-related effects due to apparently "impaired macrophage-mediated alveolar clearance" which, however, appears to be better described as an overadapted rat-specific kinetic phenomenon. It was proposed that the threshold of causing particle-induced chronic effects is the pulmonary dose that results in a first reduction in macrophage-mediated clearance due to an increase in TCC (Fig. 6).

In summary, the comparison of a modeled kinetic hypothesis with any overload-specific pulmonary inflammation delivers important pieces of information concerning the increased elimination time with increasing lung burdens. In the past, testing approaches used postexposure too short to demonstrate kinetic reversibility. Their common conclusions were to highlight the "irreversible retention of particles'" (due to the ostensibly immobilized overloaded and "enlarged" AM). Either conclusion is not supported by more recent evidence showing that reversibility can only be demonstrated if the length of postexposure

periods is adjusted to the increased elimination halftime causing the over-proportionally increased particle load and TCC. Kinetic predictions should be accompanied by proof of principle evidence as illustrated in Fig. 6 (bottom). The predicted NOAEL should empirically be demonstrated by minimal increased BAL-cell counts of controls within a range of $t_{1/2}$s of 60–90 days. At higher $t_{1/2}$s, PMNs (neutrophilic granulocytes) increase proportionally to the increased pool of migratory cells. In this context, it is important to recognize that the increase of PMNs often is caused by kinetic overload rather than any substance-specific attribute (except the agglomerate-specific density). Notably, potency comparisons of PSP require a normalization to kinetic overload (at similar elimination halftimes), otherwise any apparent substance-specific pulmonary response would be scaled to the rat-specific physiological adaption to cumulative dose rather than substance properties.

Dissolution and Bioavailability

No doubt, dissolution is tightly linked to the reciprocal relationship of particle size and surface area which determines the thermodynamic phase boundary, where the physical and chemical properties of the adjacent phases change abruptly (Pauluhn 2014a, b). As in any chemical reaction, surface processes involve breaking and making of bonds. This is what catalysis is all about. Mere reversible physical adsorption may occur; however, the adsorbent may immediately be lost by desorption, in the presence of substances with competing or stronger binding isotherms. Hence, the term "surface area−/reactivity-dependence," often preferred by toxicologists, is a highly dynamic process depending both on the kinetic and thermodynamic factors present in the intimate microenvironment of the retained particle as well as of the physical and chemical properties of the particle itself. Hence, from any modeling perspective, many surface-related factors are highly probe-of-determination-dependent. The characteristics of the probes used for the physicochemical qualification of PSP may not have any resemblance to those present in the biological system. Likewise, many simplified calculations of surface area based on idealized spherical structures cannot reliably mirror the more complex tree-dimensional assembly of nanostructures and associated void spaces.

Particle dissolution is dependent on the rate of solubilization (in mass/time), the surface area of particle and its crystallinity, or lack thereof in the case of amorphous solids. Critically, the dissolution rate may depend on the presence of other factors that determine the degree of undersaturation in the liquid solvent layer immediately adjacent to the solid solute particle. The term "interfacial solubility" was proposed to describe the average concentration of the boundary layer involved in the dissolution process of particles. Interfacial barrier models consider interfacial transport rather than diffusion through the layer. "Solubilization" is the kinetic process involving both dissolution and precipitation, which occur at the same time but in different ratios, and usually proceed by diffusion. The disjoining pressure of small particles is greater than that of large particles, so small particles have a higher interfacial solubility. Due to their higher differential

concentration, thinner diffusion layer, and increased surface area, small particles dissolve faster than larger particles. Accordingly, a thermodynamically more stable state is attained when larger particles grow at the expense of smaller particles. Thus, even if "particle disintegration" would occur it would result in thermodynamically instable conditions. Moreover, slightly soluble micron-sized particles become smaller with elapsed time, that is, before "disappearing" they transiently pass through a nanosized range. From that, a clear-cut distinction of micron- and submicron-sized PSP is difficult to make.

The solubilization kinetics, as well as apparent solubility, can be accelerated by complexation, e.g., metal ion binding by peptides or proteins present in the lung. Solubility is commonly expressed as a concentration (e.g., mass of solute per kg of solvent). The maximum equilibrium amount of solute that can dissolve per amount of solvent is the solubility of that solvent under a given condition. The advantage of expressing solubility in this manner is its simplicity, while the disadvantage is that it can strongly depend on the presence of other species in the specific microenvironment of the particle. Despite the advances of in vitro dissolution in particle qualification, the in vivo bioavailability of retained particles in the lung remains to be difficult to judge by in vitro tests. The toxicological significance of translocation and disintegration of agglomerated particles commonly ignores the complex equilibria of dissolution and precipitation processes that may occur in highly compartmentalized biological systems. For example, the solubility of any inhaled metal oxides mechanically translocated from the airways into the gastrointestinal tract may show their highest solubility in gastric fluids – due to low pH – with resultant high concentration and mucosa-to-blood gradients as the prerequisite for transport across barriers and absorption. With increasing pH following absorption, supersaturated concentrations with precipitation may ensue. Such precipitates may eventually be retrieved in the liver and spleen. Thus, caution is advised to inextricably link particles found in the extrapulmonary circulation or organs to pulmonary barrier disruption.

These elaborations demonstrate that any meaningful in vivo dissolution kinetics should use inhalation instead of instillation, insufflation, or aspiration procedures to define the fate of PSP. Likewise, to make the kinetic cornerstones comparable across different substances, any dissolution kinetics should refer to the cumulative lung burdens within a meaningful range of the threshold of kinetic overload. Typically, the dominating pathway of elimination of PSP from the lung occurs via alveolar macrophages. At yet nonoverload conditions, elimination half-times of PSP retained in the lung are in the range of $t_{1/2} = 60–90$ days as alluded to above. In case evidence of facilitated or enhanced dissolution of PM exists, a shortened halftime is observed (Pauluhn 2014).

Interdependence of Metrics on Dose

As conceptualized above, two metrics of dose must be distinguished when interrelating the pulmonary kinetics and toxicity of PSP (Pauluhn 2014). The *deposition-related metric* is frequently conceived as the conceptually better alternative to particle mass as a measure of dose would be the PSP-surface

thermodynamics. Surface area rather than mass accounts for the fact that biopersistent particles can interact only by contact of their surface, determining an effective dose-rate by a catalytic surface reaction rate that accumulates to an effective dose with increasing residence time in the target tissue. However, a finite proportion of soluble fractions of particles that may dissolve on contact with the fluids lining the airways of the lung do not contribute to "surface area dose" (apart from the fact of surface-area-dependent-facilitated dissolution). Especially for less soluble particles in a C_s-range 1–10 mg/L water, the dissolution flux is not only highly dependent on the physical characteristics of the particle itself, but also the degree of saturation of the matrix surrounding the particle has great impact. Along with these concerns, issues regarding the most appropriate unifying metric of dose are still unresolved for potentially soluble particles, of which dissolution rates increase reciprocally with PM-size. In contrast, the *retention-related metric* must observe several major variables controlling the response to any increased endocytosis of endogenous (e.g., precipitated or denatured surfactant, pulmonary phospholipidosis, and cellular debris) and exogenous poorly soluble materials. These variables include hypertrophy (enlargement commonly caused by excessive amounts of phospholipids) of the AM with minimal, if any, increase of the pool of macrophages. To the contrary, an increase of the AM pool in the absence of any hypertrophy of cells is the most common response observed in inhalation studies with PSP. At unequivocal overload, coexistence of both may occur. Typically (in rats), at yet reasonable dose rates used in repeated inhalation toxicity studies, the pool volume of AM adapts to higher PM-load by the increased influx of cells rather than their enlargement.

Distribution

Once in the bloodstream, the substance is available for distribution and elimination throughout the body as detailed in Fig. 1. Metabolism and excretion, which are components of elimination, are discussed in other chapters.

Factors that influence the rate and extent of distribution of a chemical to a particular tissue include blood flow to the tissue (rate of delivery), the mass of the tissue, the ability of the chemical to cross membranes, and the affinity of the chemical for the tissue relative to blood. The rate of distribution of a chemical from blood to tissues can be perfusion- or diffusion-rate-limited. For lipophilic chemicals that rapidly cross membranes, the rate of delivery to tissues is limited by blood flow (perfusion-rate-limited). For polar and ionized chemicals that do not readily cross the plasma membrane, the rate of delivery to tissues is limited by diffusion (diffusion-rate-limited). Plasma protein binding increases the rate of distribution to tissues for toxicants that are not diffusion-rate-limited. The free toxicant may readily cross the capillary wall, effectively decreasing its free concentration in blood. Bound toxicant then dissociates from plasma proteins to maintain the equilibrium between the bound and free forms, yet the new free molecules rapidly leave the blood, which further increases dissociation of bound toxicant, and so on. In contrast, distribution of more polar compounds that are diffusion-rate-limited is dependent on the extent of protein binding.

Initial distribution is influenced primarily by blood flow to tissues, whereas final distribution is influenced primarily by the relative affinity of the chemical for various tissues relative to blood (i.e., the tissue partition coefficient). In the early phase of distribution, tissues that receive a high blood flow (e.g., liver, kidney, and brain) may achieve high concentrations of the chemical even though the tissue partition coefficient for that chemical is low. Likewise, tissues that are slowly perfused (e.g., adipose) may achieve a low concentration of the chemical in the early phase of distribution even though the tissue partition coefficient for that chemical is very high. Later in the distribution phase, however, the chemical redistributes to tissues based on tissue partition coefficients, and the chemical is more concentrated in tissues with relatively high partition coefficients.

Excretion

In mammalian organism, several routes of elimination are available. The main excretory organ is the kidney. The kidneys receive 25% of the cardiac output; about 20% of this volume is glomerular filtration. Substances with a molecular weight less than 60 kDa are filtered by the glomeruli. As a result of this, protein-bound chemicals cannot readily be eliminated via this pathway. For effective renal elimination, lipophilic substances require biotransformation to more water-soluble metabolites, as lipophilic substances are subject to renal reabsorption, while hydrophilic metabolites may preferentially utilize active transport systems for organic anions and cations. Other excretory pathways are the gastrointestinal tract (excretion via the bile), secretory glands (e.g., milk, sweat, and tears), and, for volatile substances, the lung (Fig. 1).

Toxicokinetic Parameter

Time-Course Analyses

Toxicokinetic processes are commonly described by either zero-order or first-order kinetic profiles. When applying zero-order kinetics, the same amount of the substance, regardless of its initial concentration, is eliminated. Alcohol is a good example that is eliminated by zero-order kinetics; that means it is eliminated at a rate of about 25 mg/dL/h, a steady amount rather than a steady percentage in case of a first-order kinetics. Most substances are eliminated in something close to first-order kinetics. The biological half-life or elimination half-life of a substance is the time it takes for a substance or its metabolite to lose half of its biological activity. In a toxicological context, half-life describes the time it takes for the organ or blood plasma concentration of a substance to halve (“organ or plasma half-life”) its steady state. The relationship between the biological and plasma

half-lives of a substance can be complex depending on the substance in question, due to factors including accumulation in tissues, active metabolites, and receptor interactions. A typical example of a first-order retention and elimination kinetics has already been shown above (Fig. 5).

Volume of Distribution

The apparent volume of distribution (V_d) is a proportionality constant that relates the amount of substance in the body to its concentration in plasma. It is the theoretical volume of fluid the substance would occupy to achieve the observed concentration in plasma. For instance, a substance that is sequestered in a particular tissue will have a low concentration in plasma and a corresponding high volume of distribution, which may in fact be greater than the total body water. Thus, the volume of distribution has nothing to do with the actual volume of the body or its fluid compartments but rather involves the distribution of the drug within the body. Volume of distribution provides a reference for the plasma concentration expected for a given dose but provides little information about the specific pattern of distribution. Each drug is uniquely distributed in the body. Some drugs distribute mostly into fat, others remain in extracellular fluid, and others are bound extensively to specific tissues. Only unbound drug is available for passive diffusion to extravascular or tissue sites where the pharmacologic effects of the drug occur. Therefore, the unbound drug concentration in systemic circulation typically determines drug concentration at the active site and thus efficacy. The initial volume of distribution describes blood concentrations prior to attaining the apparent volume of distribution and uses the same formula.

Area Under the Curve

The degree of systemic exposure is defined as the integral area under the concentration-time curve, area under the curve (AUC). It represents the change in concentration over time (Eq. 4):

$$\mathrm{AUC} = \int_0^\infty C \; dt \tag{4}$$

Bioavailability (F) is the absorbed fraction of a substance according to extravascular (e.g., by inhalation, oral, or dermal), relative to the intravenous dose. The appropriate surface integrals under the plasma concentration-time curve represent the bioavailability and are dependent on many factors such as route of administration, vehicle, or species (Eq. 5).

$$\mathrm{F} = \frac{D_{iv}\mathrm{AUC}_{ex}}{D_{ex}\mathrm{AUC}_{iv}} \tag{5}$$

Significance of Toxicokinetics in Regulatory Toxicology

Toxicokinetic data are indispensable cornerstones to describe the fate, ecotoxicity, and mammalian toxicity of xenobiotics in the environment, to understand differences of their behavior in in vivo and in vitro bioassay systems, and are an essential prerequisite for risk assessment. The uncertainty involved in the extrapolation of animal-based dosimetry to humans is significantly reduced in case in-depth pharmacokinetic is available. Kinetic information is used for hazard classification and PBT (persistent, bioaccumulative, toxic) assessment as well as wildlife and human food chain exposure modeling for the chemical safety assessment. It is also a factor in deciding whether long-term toxicology testing might be necessary. This is because chemical accumulation may result in internal concentrations of a substance in an organism that causes toxic effects over long-term exposures even when external concentrations are very small. Highly bioaccumulative chemicals may also transfer through the food web, which in some cases may lead to biomagnification.

The expression of toxicity arising from exposure to a substance is a consequence of a chain of events that results in the affected tissues of an organism receiving the ultimate toxicant in amounts that cause an adverse effect. The factors that confer susceptibility to certain species and lead to major differences between animals and humans, in their response to such chemical insults, are based either on the nature and quantity of the ultimate toxicant that is presented to the sensitive tissue (toxicokinetics, TK) or in the sensitivity of those tissues to the ultimate toxicant, that is, the toxicodynamic response. While their toxicokinetic data are mandatory for pharmaceuticals, there is no specific requirement to generate toxicokinetic information in the notification or authorization process of chemicals. Nonetheless, in the European REACH regulation, Annex I states that "the human health hazard assessment shall consider the toxicokinetic profile (i.e., absorption, metabolism, distribution, and elimination) of the substance." Likewise, REACH announces in Annex VIII that one should perform "assessment of the toxicokinetic behavior of the substance to the extent that can be derived from the relevant available information."

Although TK is not a toxicological endpoint and is not specifically required by chemical regulations, the generation of TK information is definitely encouraged as a means to better interpret and amalgamate data from different sources and to assist testing strategy and study design, as well as category development, thus helping to optimize test designs: Prior to any animal study, it is crucial to identify the benefits that will be gained from conducting such a study. The TK behavior derived from available data might make further testing unnecessary in terms of predictability of other properties. The most critical factor influencing toxicity is the concentration of the ultimate toxicant at the actual target site (tissue dose). In this context, bioavailability is a relevant parameter for the assessment of the toxicity profile of a test substance. It links dose and concentration of a substance with the mode of action, which covers the key events within a complete sequence of events leading to toxicity.

The definition of actual TK studies on a case-by-case basis might further improve the knowledge about substance properties in terms of expanding knowledge on properties sufficiently to enable risk assessment. Overall, the formation of data that are unlikely to be used and that constitute an unnecessary effort of animals, time, and resources shall be

avoided using any supporting data to do so. Moreover, it can provide important information for the design of (subsequent) toxicity studies, for the application of read-across and building of categories. Taken together, along with other approaches, TK can contribute to reduction of animal use in toxicology and reduces uncertainty in risk assessment.

Future Directions

With the advent of nanotechnology, nanostructures are increasingly investigated in in vitro cell culture systems. Particokinetics need to be improved and refined for insoluble structures for comparative in vivo/in vitro cellular dosimetry. Toxicokinetics may be playing a more significant role in predictive in silico toxicology with PBPK-modeled study design to illustrate the complex relationship between toxicity and physicochemical characteristics which make generalizations possible with less numbers of experimental animals. Theragnostic (a portmanteau of *therapeutics* and *diagnostics*) is a proposed process of a targeted diagnostic therapy for individual patients for new principles of medication and to tailor treatment by a combination of pharmacokinetics and pharmacodynamics.

Cross-References

- ▶ Importance of Physicochemical and Physical Properties for Toxicological Risk Assessment
- ▶ Medical Aspects of Traffic Exhaust Regulations
- ▶ Nanoparticles and Their Regulation
- ▶ Single Substances Versus Combined Effects: Problems in Mixture Toxicology
- ▶ Susceptible Population Groups in Regulatory Toxicology
- ▶ Toxicokinetic Models

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Toxicodynamic Tests

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Alexander Cartus and Dieter Schrenk

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A. Cartus · D. Schrenk (✉)
Food Chemistry and Toxicology, University of Kaiserslautern, Kaiserslautern, Germany
e-mail: cartus@chemie.uni-kl.de; schrenk@rhrk.uni-kl.de

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Abstract

Toxicodynamic testing is aimed at the elucidation of adverse effects of chemicals including understanding of their mode of action. In many cases, the "standard program" of toxicological testing on acute, subchronic, or chronic toxicity, genotoxicity, carcinogenicity, teratogenicity, and developmental and reproductive toxicity, which is needed for many regulatory purposes, already provides important information on the mode(s) of action of a compound. Targeted mechanistic investigations often follow, which use specifically designed models such as genetically modified cells or animals, studies using specific cell types, subcellular fractions, enzymes, etc. The understanding of the mechanisms underlying a certain mode of action and gained information on the dose- or concentration-response from in vivo or in vitro studies is crucial to derive point of departures for further human risk assessment and for regulatory toxicology of chemicals since it allows decisions on the options for extrapolation of experimental data to the human situation. This text follows the different levels of experimental models in toxicodynamic testing from isolated target molecules up to whole organisms like laboratory animals and humans.

Keywords

Toxicity testing · Mode-of-action · Toxicodynamics · Mechanism-of-action · In vitro

Introduction

Depending on the special toxicological question addressed (Gregus and Klaassen 2001; Hayes 2001; Krewski et al. 2020), experimental models in toxicodynamic testing can make use of different hierarchical biological stages beginning with isolated target molecules like enzymes or receptors up to whole organisms like laboratory animals and humans as depicted in Fig. 1. Of course, biological complexity increases in this direction, possibly along with other factors like availability, price, or ethical issues or hindrances. In the following sections, we summarize and discuss options, advantages, and disadvantages of different experimental models in toxicodynamic testing.

Isolated Target Molecules

Nucleic Acids

Isolated nucleic acids of various degrees of purification can be obtained from different sources (DNA from calf thymus, herring sperm, tissue cultures, etc.) and be incubated with a chemical and/or its metabolites to detect if covalent binding occurs which may implicate a genotoxic/mutagenic mode of action. Reactive metabolites can be generated in situ by adding activating enzymes ("S9 mix") to the

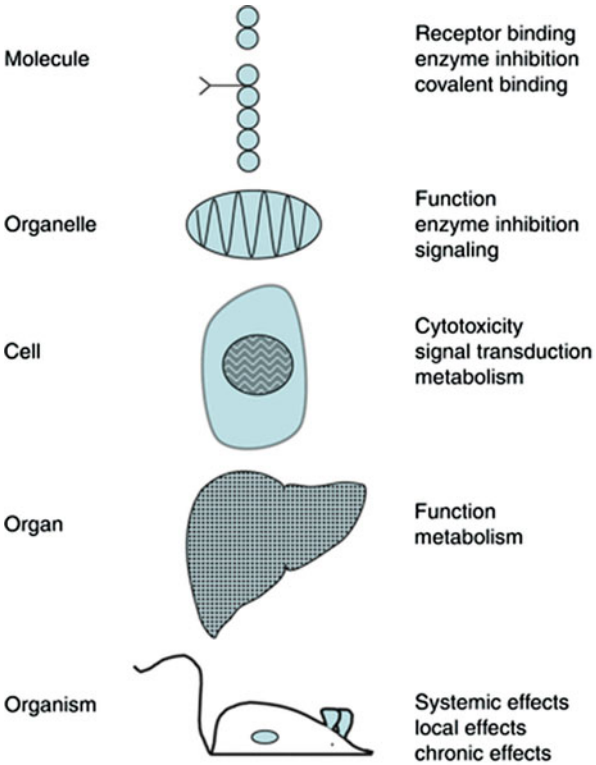

Fig. 1 Levels of experimental models in toxicodynamic testing

incubation. Subsequently, the nucleic acids are extracted, digested, and analyzed, e.g., for covalent binding of nucleosides to the chemical and/or its metabolites. Stable isotope-labeled DNA adduct standards can be added to the nucleoside preparation to quantify known DNA adducts most frequently via sensitive and specific LC-MS/MS methods. Alternatively, nucleotides (after hydrolysis) including modified nucleotides can be post-labeled with radioactive ^{32}P containing phosphate for further separation and identification via autoradiographic TLC (^{32}P-Postlabeling) which may help to screen for structurally unknown DNA adducts. Also, epigenetic alterations can be investigated in cell lines or in vivo, e.g., DNA methylation pattern or several histone (protein) modifications in intact DNA.

Proteins/Enzymes

The chemical or material of interest can be incubated with tissue or cell homogenates or with purified enzymes or other proteins. Assays are aimed at testing covalent or noncovalent binding but also functional effects on proteins (Pumford and Halmes 1997). Well-known examples are the inhibition of acetylcholine esterase by

organophosphates, binding of inhibitors of mitosis to tubulin in the spindle apparatus, or enzyme inhibition by certain heavy metals such as mercury ions. In the course of such tests, information on the type of inhibition can be derived from concentration-effect analysis using a variety of inhibitor concentrations.

Lipids

Incubating purified lipids with test compounds or their metabolites can also be used to identify possible covalent or noncovalent binding or, for example, to study lipid peroxidation (via end products like malondialdehyde or 4-hydroxynonenal) as a marker for oxidative stress in a cell or organism (e.g., TBARS (thiobarbituric acid reactive substances) assay). Again, addition of an enzyme preparation can be used to modify, e.g., activate, the test compound in purified preparations.

Subcellular Fractions/Organelles

Membranes: Cytoplasmic Fraction

The common method to isolate membrane fractions is sequential centrifugation. Likewise, a total membrane fraction can be isolated from a liver homogenate ultracentrifugation at 100,000 × *g*, after nuclei, mitochondria, etc. have been sorted out at lower *g* numbers. The supernatant of the membrane fraction represents the soluble cytosolic fractions, sometimes called "cytosol." The sediment ("membrane fraction") can be resuspended and subjected to additional (gradient) centrifugation in order to enrich certain types of membranes. Following this approach, fractions enriched in endoplasmic reticulum ("microsomes") – or outer cellular membrane-derived membranes – can be prepared. The degree of enrichment can be verified by measuring the presence or activity of marker proteins after addition of needed cofactors.

Such fractions can be used for the investigation of membrane-bound (CYPs, UGTs, etc.) or cytoplasmic (GSTs, STs, etc.) enzyme activities, induction, inhibition, etc. Furthermore, the metabolism of chemicals including genotoxic carcinogens, leading eventually to mutagenicity, DNA binding, etc., can be analyzed. Together with kinetic parameters obtained from such time- and/or concentration-dependent experiments, also biokinetic properties and eventually DNA-binding activities in vivo can be estimated (physiologically based biokinetic, PBBK modeling). The supernatant of a 9000 × *g* centrifugation of homogenized liver is called S9 mix or S9 fraction, which contains microsomes and cytosol.

Receptors

In a strict sense, receptors act as triggers of signaling chains responding to agonistic molecules by binding and change in receptor conformation. A typical consequence of receptor activation is the formation of intracellular signal molecules called

"second messengers." Likewise, the binding of noradrenalin to $ß_1$-adrenoceptors can result in enhanced intracellular formation of the second messenger cAMP. Xenobiotic chemicals can act on both membrane-bound receptors on the outer cellular membrane and on intracellular receptors, being located, e.g., in the cytoplasm or the nucleus. Also, trafficking of activated receptors, i.e., translocation from the site of ligand binding to the site of effect, is common. Xenobiotic ligands can mimic endogenous ligands, thus activating receptors thought to be responsive to hormones, transmitters, etc. In some cases, endogenous ligands are unknown ("orphan receptors"), or there is no scientific agreement on the identity of "the endogenous ligand" although a variety of endogenous compounds can bind to the receptor.

Effects of xenobiotic chemicals on receptors have been widely described and are considered as a central field in toxicodynamic research. In many instances, such effects are wanted, representing a fundamental mode of action of many therapeutic drugs. In toxicology, receptor activation can be crucial for many adverse effects. One example is the activation of hormonal receptors like ER (estrogen receptors), allowing to determine the "endocrine-disrupting" effect of chemicals in a direct receptor-binding assays or via reporter gene assays for chemicals that otherwise may not have toxicological adverse effects (like being genotoxic or carcinogenic, etc.). Also, the binding of dioxins to the aryl hydrocarbon receptor (AhR) is a prominent example. A major field of research on xenobiotic-responsive receptors is the adaptive response of drug-metabolizing enzymes called "induction of drug metabolism." This phenomenon, which can have adverse consequences for the organism, is used as a marker for certain types of receptor activation being monitored as a regulated battery of genes/enzymes. Some important examples for such concerted responses are given in Table 1 listing, e.g., the AhR, CAR (constitutive androstane receptor), PXR (pregnane X receptor; Fig. 2), or the PPARs (peroxisome proliferator-activated receptors).

Ligand binding to the receptor can be agonistic, partially agonistic, or antagonistic. This classification can depend on receptor subtype, cell type, species, etc. Furthermore, a compound can bind to an alternative ("allosteric") binding site on the receptor, thus modulating the affinity and/or effect transmission capacity of the "real" ligand which binds to the ligand binding site. These phenomena can be studied including binding assays in receptor-enriched tissue fractions or transfected cell lines which (over-)express the receptor of interest, e.g., combined with a specific reporter gene construct.

Transfer Through Biological Membranes (Ion Channels, Transporters, and Pumps)

In most cases, the function of ion channels, transmembrane transporters, and pumps is investigated using membrane fractions since most of these proteins are embedded in membranes. From the latter, vesicles can be prepared which can be used for transport studies, e.g., with radioactively labeled transport substrates. Such models are suitable for the analysis of the binding affinity of standard substrates, modulation of transport function, properties of a test compound as transport substrate,

Table 1 "Xenobiotic" receptors regulating expression of drug-metabolizing enzymes

Receptor	Chemical/compound	Inducible enzyme(s)
Aryl hydrocarbon receptor (AhR)	DL-PCBs, PAHs, TCDD	CYP1A1, 1A2, 1B1
Constitutive active (androstane) receptor (CAR)	DDT, NDL-PCBs, phenobarbital,	CYP2B1, 2B2, 2B6, UGT2
Peroxisome proliferator-activated receptors (PPARs)	Fibrates, phthalates (diethylhexyl phthalate)	CYP4A
Pregnane X receptor (PXR)	Clotrimazole, dexamethasone (rodents), HBCD, pregnenolone 16α-carbonitrile, rifampicin	CYP3A, 7A1, OATP2, MRP2, MDR1/Pgp
Nrf2 (via antioxidant-responsive element; ARE)	BHA, BHT, t-butylated hydroquinone	GSTYa, M, P1, NQO1

Abbreviations: *BHA* butylated hydroxyanisole, *BHT* butylated hydroxytoluene, *CYP* cytochrome P450, *DL-PCB* dioxin-like polychlorinated biphenyls, *GST* glutathione *S*-transferase, *HBCD* hexabromocyclododecane, *MRP* multidrug resistance-associated protein, *MDR/Pgp* multidrug resistance protein/P-glycoprotein, *NQO* NAD(P)H-quinone oxidoreductase, *NDL-PCB* non-dioxin-like polychlorinated biphenyls, *OATP* organic anion transporter, *PAH* polycyclic aromatic hydrocarbons, *TCDD* 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, *UGT, UDP*-glucuronosyl transferase

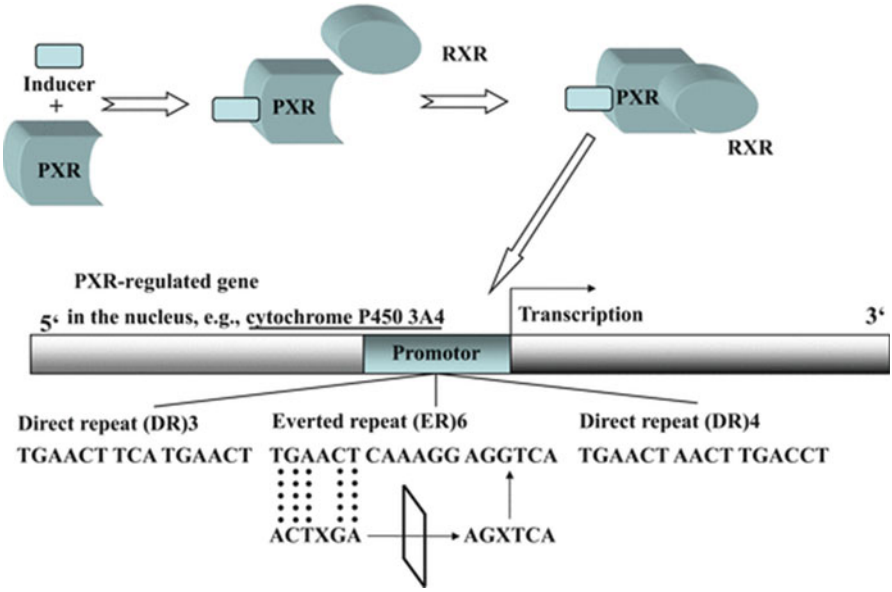

Fig. 2 Induction of gene expression via the pregnane X receptor (*PXR*). Upon ligand binding, the receptor dimerizes with the retinoid X receptor (*RXR*). The dimer binds to consensus sequences (direct repeats, inverted or everted repeats) in the 5′-flanking region of responsive genes, thus modulating their transcription

conformational changes in protein structure upon substrate binding, etc. Furthermore, cell cultures can be applied in order to investigate the consequences of a targeted overexpression of a certain transmembrane protein, its genetic elimination ("knockout"), or selective inhibition by antagonists.

Finally, transmembrane transfer proteins can be regulated at the level of gene expression and localization within the cell ("trafficking") or tissue, in tissue culture or whole organisms.

Mitochondria

Mechanistic studies in isolated mitochondria comprise the investigation of mitochondrial damage (loss of physiological function) and mitochondrial signaling. Mitochondrial enzymes involved in oxidative phosphorylation/ATP production and oxygen consumption ("respiratory chain") are typical targets of chemicals (blocking of respiration, uncoupling of oxygen consumption and ATP formation, etc.). Signaling compounds released by damaged mitochondria comprise cytochrome c, calcium ions, and many others. Gross change in mitochondrial function can be measured as changes in membrane potential, proton concentrations, oxygen consumption, calcium flow, ATP/ADP ratio, etc.

Nuclei

Isolated nuclei are used for mechanistic studies investigating effects of chemicals on gene transcription (nuclear run-on assays), covalent and/or noncovalent ("intercalation") binding to DNA, other types of DNA damage (e.g. by oxidation, strandbreaks), and modifications of chromatin and effects on nucleosomes or on DNA/chromatin processing enzymes (topoisomerases, nucleic acid polymerases, etc.).

Cells

Permanent Cell Lines

In contrast to many primary cells in culture, permanent cell lines always proliferate in culture being harvested from the culture plate and seeded onto empty cell culture dishes. This "passaging" can virtually be used as an infinite source of cells. However, permanent cells frequently change their properties after several rounds of passaging. Thus, the passage number should be provided as an additional source of information in experiments with permanent cells and tests with high passage numbers should be avoided.

Permanent cell lines are of limited use in the study of the mode of action of a chemical because they usually differ more or less from the corresponding primary

cell type. In many instances, permanent cell lines are derived from tumors exhibiting profound changes in genotype and phenotype when compared to normal cells. For the successful use of permanent cells lines, their properties should be investigated as far as possible. A focused analysis of effects on defined signaling pathways, which are known to be regulated in a similar way in primary cells, is a typical example for such use.

For instance, it has to be considered that ATP production in many classical proliferating cancer-derived cell lines is based on glycolysis under hypoxic conditions rather than oxidative phosphorylation which decreases cells susceptibility to mitochondrial toxicants. Thus, to study mitochondrial toxicity, glucose in the cell culture medium may be replaced by galactose to increase mitochondrial activity. Another important issue is to ensure that enzymes/transporters for the uptake of a chemical that has to be examined or enzymes to metabolize a chemical, e.g., to a mutagenic electrophile, are expressed and active in a used cell line. However, genetically modified permanent cells lines can be a well-suited tool to investigate several toxicological endpoints if it is warranted that all necessary enzymes are produced. Furthermore, genetically engineered permanent cell lines over- or under-expressing certain genes of interest provide a powerful tool to study the influence of the encoded proteins on various outcomes, pathways, etc.

Primary Cells and Organoids

Cells isolated from certain organs or tissues of humans or experimental animals such as liver, lung, kidney, or immune cells usually comprise a mixture of several cell types. The cell preparations are obtained, e.g., by perfusion of the organs with media which disintegrate the tissue or by lavage of the organ surface (e.g., pulmonary epithelia). Individual cell types, e.g., hepatocytes (liver), alveolar cells type I (lung), or macrophages (blood, tissues), can be prepared from mixtures of different cell types by sequential centrifugation/density gradient centrifugation. Many primary cell types can be seeded and adhere on uncovered or specifically covered cell culture dishes or tissue culture flasks. The culture conditions usually aim at keeping the cells as long as possible in their differentiated state, i.e., to maintain their tissue-specific ("in situ") properties and functions. In most instances, this aim cannot be achieved completely, and/or differentiation is partially lost during culture. Usually, permanent cells undergo senescence or lose their specific phenotype after a certain time in culture. This can partially be circumvented using 3D embedding or suspension techniques using extracellular matrix. Beside this, generating organoid structures from adult or pluripotent stem cells is a promising tool to study organ toxicity in a model near to the in vivo situation; however such models are not always commercially available (Messina et al. 2020). Parameters which allow conclusions on the mode of action of a chemical in cell cultures include cytotoxicity and cell death, effects on cell culture density, proliferation, apoptosis, as well as changes in protein synthesis or growth behavior (e.g., loss of contact inhibition, growth in soft agar). Likewise, the mechanisms leading to necrosis or apoptosis in cell culture are

investigated in detail (Wyllie 1997). Hallmarks of molecular pathways are activation of receptors (Fas receptor; TGF-ß1 receptor, etc.), mitochondrial signaling, changes in apoptosis-regulating factors (TNF alpha, bcl-2, bax, p53, etc.), or activation of caspases. In such investigations, various cell types equipped with different receptors as well as various derivatives of the test compound can be used. Furthermore, "omics" analyses detecting changes in gene expression (gene arrays, etc.), protein patterns (proteomics), and endogenous metabolites (metabonomics) play a more and more important role in identifying the cellular mode of action of a chemical but also need bioinformatic methods for their analysis due to the large amount of gained data. In more specific studies, secretion of certain growth factors or tissue hormones, matrix-cell interactions, release of transmitters, etc. are analyzed. The effects of such changes can be measured directly in co-cultures with respective responder cells (e.g., immune cells). In addition, certain biochemical effects such as enzyme inhibition, binding to nucleophilic targets, generation of reactive oxygen species, etc. can also be analyzed in primary cell cultures.

Of particular interest in toxicology is the investigation of genotoxic events in primary cells. These analyses comprise the determination of modified DNA bases, DNA fragmentation, mutations, micronuclei formation, chromosomal changes, DNA repair, etc.

Tissues

Isolated Organs

Isolated perfused organs such as the liver, lung, heart, intestine, or kidney from rat, rabbit, or guinea pig represent widely used models for the study of the mode of action of a chemical in toxicological research. They allow, e.g., the study of necrotic cell damage and its modulation by inhibitors of metabolic activation or by the addition of protective substances (e.g., of acetylcysteine in paracetamol-mediated liver damage). Furthermore, the issue of localization of the damage or of the underlying biochemical pathway can be addressed. Likewise, perfusion with an acute nephrotoxicant allows the determination of the exact site of tubular damage or the role of glutathione depletion in such a scenario. The perfusion rate (flow) and pressure characteristics can be of interest in analyzing the pathogenesis of a damage, e.g., in particular in lung or kidney. In addition, "functional" effects in an isolated organ such as changes in heart rate, uterus contraction, etc. can be detected. The duration of experiments with isolated organs is limited by the lifespan of the organ being between minutes and a few hours. In many cases, this time is sufficient, however, to obtain relevant amounts of metabolites from a chemical or sufficient organ damage, depending on the start concentration of substrate. A novel development in tissue research is the use of organs isolated from domestic animals such as pigs or cows from slaughterhouses. This method allows the reduction in numbers of experimental animals and benefits from the relatively close relationship between porcine and human physiology when compared to rodents.

Tissue Slices

Studies in tissue slices allow one to address many questions which can also be dealt with in isolated perfused organs or in cell culture. Thus, this model is positioned between cells and intact organs. Tissue slices are easy to prepare and use (no difficult preparation, no perfusion equipment, etc.) but lack the physiological perfusion via the blood vessels. Nevertheless, tissue slices in certain instances may allow relevant conclusions about the type of tissue damage, xenobiotic metabolism, and its modulation or complex changes in gene expression.

In Silico Methods

In silico methods are aimed to complement existing toxicity tests to predict toxicity, prioritize chemicals, guide toxicity tests, and/or minimize late-stage failures in drugs design. Those methods are interesting, because they are faster and cheaper than in vitro or in vivo experiments and of course for ethical reasons because no animal experiments are needed. As mentioned before, e.g., toxicokinetic parameters can be obtained by PBBK modeling using in vitro data. Also, for some toxicodynamic endpoints in silico methods are somewhat useful or are even already accepted in some regulatory fields. Methods include knowledge-based (i.e., decision trees have to be completed guided by rules defined by experts), QSAR models (quantitative structure-activity relationship; using a set of chemicals with known effect to span a domain in which the unknown chemical is inter- or extrapolated using different determinants) and read-across methods (based on structural similarities). The most developed endpoint in this regard is probably genotoxicity/mutagenicity, whereas other endpoints may be rather poorly predictable yet. For example, the risk assessment of genotoxic impurities in pharmaceuticals can be performed using different in silico methods (at least one knowledge-based and one QSAR model) and the TTC approach (threshold of toxicological concern) under EMAs ICH M7 guideline. Other endpoints/mode of actions that can currently be evaluated with different quality include DNA and protein reactivity, metabolism by cytochrome P450 and phase II enzymes, skin sensitization, or even carcinogenicity (genotoxic/non-genotoxic). Free and proprietary software tools are ToxTree, QSAR toolbox, Lhasa Nexus, Vega, and many others.

Adverse Outcome Pathways

An adverse outcome pathway (AOP) describes a series of so-called key events (KE) linked by key event relationships (KERs) on many hierarchical stages (from the molecular level to a whole organism) that are necessary to develop a toxicological adverse outcome, i.e., a disease or an effect like skin sensitization, followed by a molecular initial event (MIE). An important assumption of AOPs is that toxicological processes tend to share KEs and KERs, within an individual organism and also

across species. Furthermore, one MIE can be associated with different adverse outcomes and vice versa (Krewski et al. 2020). In case of skin sensitization, the MIE (after absorption) is the covalent reaction of a chemical with skin proteins, and KEs are keratinocyte response (activation of inflammatory cytokines), mobilization of dendritic cells and T-cell proliferation. All of these MIE or KEs are separately assessable with in vitro methods (see OECD Test guidelines: 428, 442C. 442D, 442E, 429). Together with the development of further specialized in vitro assays which address single KEs and MIEs as alternatives to animal testing and with in silico methods, assessment of AOPs may play a major role in the future in reducing and replacing animal experiments in line with the 3R concept.

Experimental Animals

Acute Toxicity/Organ Toxicity

Experimental animals represent the most relevant model for the comprehensive prediction of adverse effects of chemicals in humans. Also studies on the mode(s) of action of a chemical can be performed in animals covering many various aspects. For example, the effects of a chemical on certain enzyme activities, levels of hormones, growth factors, etc. in blood or target tissues can be investigated. Furthermore, a broad spectrum of parameters of organ function and morphology (histopathological analysis) can be carried out. From the complex picture thus obtained, conclusions can be drawn on the possible mode of action. These can be substantiated by the target application of modulators such as enzyme inhibitors. Furthermore, studies on effects on gene expression and transcription ("genomics/transcriptomics"), protein levels ("proteomics"), endogenous metabolites ("metabonomics"), or the metabolism of the xenobiotic chemical of interest ("metabolomics") are essential parts of the current broad approach in toxicological research.

Using modern methods of genetic engineering and breeding, genetically modified strains can be obtained which allows further conclusions on molecular targets. Examples are rodent strains with deleted or silenced genes ("knockout" animals) or strains which overexpress a certain homologous or heterologous ("humanized") gene. Likewise, the study of Ah receptor-knockout mice has provided crucial insight into the biology of this receptor and its role in dioxin toxicity. Another example is the use of DNA repair-deficient mice to investigate the role of DNA repair mechanisms on the genotoxicity of chemicals.

Chronic Toxicity/Organ Toxicity

The investigations (and prediction) of chronic adverse effects, i.e., lifetime exposure, e.g., over a period of 1–2 years for a chronic rat study with daily (or 5 days/week) treatment of a chemical via the appropriate route (oral, dermal, inhalation), represent

the most challenging task in toxicological research (see ▶ Chap. 9, "Examination of Acute and Repeated-Dose Toxicity"). The relevant changes are mostly unknown when the experiment starts. Furthermore, exposure in a certain time window may be the most relevant. In any case, animal experiments still are the most reliable tool in predicting chronic toxicity in humans. Crucial endpoints can be clinical, (histo) pathological, biochemical observations including weight gain, food/water consumption, organ weights, hematological changes, mortality rates, any morbidity and histopathological changes in organs, etc. For a more comprehensive overview, see OECD Test guideline 452 (Chronic Toxicity Studies). Accompanying in vitro studies can be applied to obtain more information on the molecular mechanisms or mode of action underlying adverse effects observed in chronic animal studies.

Other Modes of Action

Targeted analyses in animal testing are aimed at understanding mode(s) of action. They make use of the broad pattern of biochemical and pharmacological testing approaches such as changes in intestinal passage, blood flow, arterial blood pressure, bile flow, renal blood flow, and inulin clearance, to mention a few. However, a minor temporal change in bile flow or blood pressure does not necessarily represent an adverse effect since it also occurs under physiological conditions representing reversible, adaptive responses (see ▶ Chap. 43, "Adverse Effects Versus Non-adverse Effects in Toxicology"). Such observations can be very helpful, however, in the understanding of a mode of action and may even be useful in the development of new therapeutic drugs. Additional experiments frequently follow in order to clarify the molecular mechanisms leading to the observed mode of action, e.g., an induction of a biliary export pump in increased bile flow. The induction of drug-metabolizing enzymes is another example of a frequently observed, adaptive, and thus not necessarily adverse consequence of xenobiotic exposure in laboratory animals.

Genotoxic and Carcinogenic Effects

Mechanisms of genotoxic effects can be found in many of the aforementioned experimental models. Following the paradigm that mutagenic effects and primary carcinogenic ("initiating") lesions are permanent changes in nuclear DNA, the investigation of genotoxic events is focused on DNA. They include bacterial (Ames test, rec test) or yeast cells, mammalian cell lines (sister chromatid exchange, micronucleus test, HPRT assay, comet assay, etc.), or intact animals (mouse micronucleus assay) identifying DNA strand breaks, mutations, and aneugenic or clastogenic effects.

The enormous complexity of the carcinogenic process does not allow a comprehensive testing for carcinogenicity using short-term assays. Phenomenologically, carcinogenicity can be studied using laboratory animals (OECD Test guideline 451), most often rats and/or mice or non-rodent species. Although the main reason to conduct such a study is to obtain information on tumor formation and incidences in

different organs, data as mentioned for chronic studies (e.g., weight gain, hematological, clinical, biochemical data etc.) are collected as well. The multistage concept of carcinogenesis suggests the existence of a primary lesion, which predisposes the "initiated" cell for a development into a malignant cell passing various stages. These stages, also termed as promotion and progression, require the presence of additional factors which allows the cell to proceed on this way. It is unclear if these additional steps involve or even require specific genetic changes. Furthermore, predisposing genetic changes in "normal" cells may make those cells vulnerable to additional factors and may even be inherited by the organism. Examples for such predispositions are the familial polyposis coli with respect to colon cancer or the hereditary disposition for breast cancer. A widely used tool to investigate the multistage development of cancer is hepatocarcinogenesis in rodents. In this model, certain mutations in critical genes (hot spots), e.g., in the H-Ras proto-oncogene, are linked to the initiation step (Anderson et al. 1992). The subsequent phase of promotion can be facilitated by chemical factors (tumor promoters) which may inhibit apoptosis of initiated cells, e.g., by suppression of pro-apoptotic pathways or by inhibition of intercellular signaling, etc. Likewise, certain receptors, such as CAR (constitutive androstane receptor), PPAR alpha (peroxisome proliferator-activated receptor alpha), ER (estrogen receptors), and GHR (growth hormone receptor), can mediate the promotion effect. Detailed studies, e.g., with humanized mice have led to the suggestion that receptor-mediated liver tumor promotion, e.g., with phenobarbital, can markedly differ between rodents and human, depending on receptor-mediated signaling. These studies illustrate the difficulties in the use of rodent-derived tumor-promotion data in regulatory toxicology.

Teratogenicity and Developmental and Reproductive Toxicity

These investigations make use of almost all aforementioned experimental models using subcellular, cellular, organ, tissue, or whole animal systems. In addition to animal experiments in rodents, birds, and amphibians, mechanistic studies are aimed at the role of receptors (retinoid receptors, PPARs). Exposure of dams during pregnancy/lactation does ideally not lead to maternal toxicity. While malformations are frequently seen after birth, developmental effects can occur at later life stages or even only become visible at more advanced stages (learning behavior, etc.) or when the fertility of the offspring is investigated ("multi-generation study"). Detailed studies on reproductive toxicity of a chemical in experimental animals comprise macroscopic and microscopic investigation of changes in the reproductive organs, reproductive behavior, perturbations of steroid hormone homeostasis and metabolism, receptor-linked effects, etc. including an analysis of fertility and reproductive success. OECD guideline tests to study developmental and reproductive toxicity include the test guidelines 414 (Prenatal Developmental Toxicity Study), 416 (Two-Generation Reproduction Toxicity), 421 (Reproduction/Developmental Toxicity Screening Test), and 443 (EOGRTS: Extended One-Generation Reproductive Toxicity Study).

Alternatives to Animal Tests

In line with the 3R concept (replacement, reduction, refinement of animal testing) introduced by Russell and Burch already in 1959, many toxicity tests for certain endpoints/modes of action prior partly or solely performed using laboratory animals are nowadays replaced by in vitro and/or in silico methods as described above. Examples are, for example, the BCOP (Bovine Corneal Opacity and Permeability) assay for eye irritation or the 3 T3 NRU Phototoxicity Test. A complete, frequently updated status report on validated and accepted alternative methods can be obtained from EURL-EVCAM (2018).

Investigations in Humans

Toxicodynamic studies in humans include those during development of new drugs. Here, pharmacological studies can provide information on possible unwanted/adverse effects. Furthermore, interferences of chemicals with the signaling or metabolism of other compounds or substrates including endogenous compounds are of interest. In the field of receptors and drug-metabolizing enzymes, genetic polymorphisms have been identified in humans such as polymorphisms in the CYP2D6, CYP2C19, NAT2, GST μ, genes, etc. These can result in toxicokinetic effects on the fate of chemicals which may have strong implications for the toxicodynamics. The methods used to identify those polymorphisms comprise DNA investigations looking for individual point mutations or more frequent single nucleotide polymorphisms, as well as gene expression analysis such as RT-PCR, western blotting, enzyme assays, or next-generation sequencing methods. Metabolism tests in healthy human volunteers are widely used to investigate the consequences of genetic polymorphisms of this type on the kinetics of standard substrates such as caffeine (CYP1A2, NAT2), debrisoquine (CYP2D6), or chlorzoxazone (CYP2E1) (Keller et al. 2017).

Experimental studies on chemicals other than drugs have been carried out in human volunteers under strict ethical and technical rules aiming at the prevention of severe or sustained adverse health effects in the cohort. Epidemiological studies, both observational and interventional, can provide valuable additional information on possible correlations between exposure and adverse outcome in humans. These usually require, however, strong support from biochemical, cell culture and animal data to reach the level of causality.

Cross-References

► Adverse Effects Versus Non-adverse Effects in Toxicology
► Aims and Mission of Regulatory Toxicology
► Epidemiological Methods in Regulatory Toxicology
► Examination of Acute and Repeated-Dose Toxicity

- ▶ International Regulation of Toxicological Test Procedures
- ▶ Omics in Toxicology
- ▶ Specific Toxicity Tests for Neurotoxicity, Immunotoxicity, Allergy, Irritation, Reprotoxicity, and Carcinogenicity
- ▶ Toxicity Testing In Vitro: Regulatory Aspects

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Omics in Toxicology

18

Heidrun Ellinger-Ziegelbauer and Hans-Juergen Ahr

Contents

Abstract

The tremendous progress in the development of new technologies in the areas of molecular biology and bioinformatics enables interrogation of cellular responses to toxicant treatment at a global molecular level, allowing evaluation of toxic effects in the context of molecular pathways.

The major techniques currently employed, especially transcriptomics, but also proteomics and metabolomics, are being used and further evaluated in investigational toxicology. Since they already have been shown to provide increased insight into molecular mechanisms of toxicological effects, such data have been submitted to regulatory authorities to support regulatory assessment of new

H. Ellinger-Ziegelbauer (✉) · H.-J. Ahr
Investigational Toxicology, Bayer AG, Wuppertal, Germany
e-mail: heidrun.ellinger-ziegelbauer@bayer.com

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compounds in few cases. Still, such data could be used more broadly for hazard identification and even risk assessment, which is now being supported by recent initiatives though precompetitive collaborations.

Keywords

Omics · Genomics · Toxicogenomics · Proteomics · Metabolomics · Mechanistic · Predictive toxicogenomics · Signatures · Liver cancer

Introduction

Advancements in molecular biology research, especially the development of the microarrays in the 1990s, allowed the development of new technologies to perform detailed analyses of fundamental processes in living organisms. These so-called omics technologies enable the simultaneous measurement of all definable entities of an "-ome," corresponding to a certain class of molecules in biology. Due to rapid development of DNA sequencing techniques, many genomes have now been completely sequenced, including human, mouse, and rat (genomics). Parallel analysis of all expressed genes in an organ or cell at the mRNA (transcriptomics) and protein (proteomics) level provides much increased insight into biological processes at the molecular level. Recently, similar analyses of noncoding RNAs, including long-noncoding RNAs (lncRNA) and microRNAs (miRNA), have been added as omics tool. Finally, measurement of all metabolites in cells and tissues or in body fluids indicates functional changes of cellular metabolism (metabolomics).

In general, the different omics technologies deliver complementary data. One technology is likely not sufficient to reveal all molecular processes interacting at the cellular or organ level (Fig. 1). Yet even if just one technology is used, it does

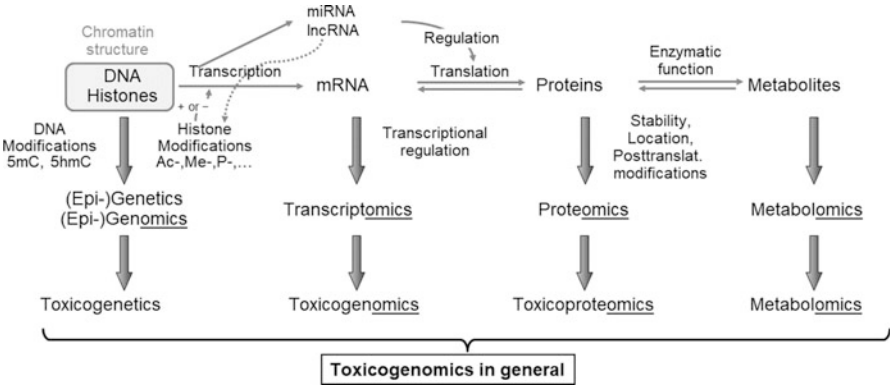

Fig. 1 Omics technologies, applied in toxicology for global analyses of the major molecules present in biological samples, represent the major levels of gene expression and cellular pathways. The term "toxicogenomics" may also be used as a general term for omics applied to toxicological studies

increase our knowledge of toxicological processes. Currently, the technical requirements and maturity of the individual technologies are different.

Toxicogenomics

Measurement and analysis of gene expression profiles (transcriptomics) under the influence of chemical stressors or toxic compounds (toxicogenomics) or in disease states is technically the most advanced among omics technologies. This is due to the underlying low complexity of the molecules to be measured, which are represented by a combination of only four bases obeying to clear principles of complementary base pairing, and to the relatively simple chemistry behind these molecules being experimentally easily accessible. Efficient techniques for quantitative measurement of the expression levels of many genes in a single sample could therefore be developed.

The first methods for global expression profiling were relatively elaborate open-profiling methods like *differential display*, which are based on conversion of all mRNAs in a sample into cDNA and extensive further processing to display differential expression levels. These are hardly used anymore. The emergence of whole genome *microarrays* represented a breakthrough then enabling routine whole genome profiling analysis. Microarrays allow interrogation of the expression level of essentially all known genes and/or sequenced transcripts of a species of interest.

In the beginning's microarrays were available in different designs, yet a few major ones are used nowadays, including arrays based on oligonucleotides, either printed on a solid support, attached to beads, or synthesized in situ onto a wafer chip. These types of arrays can be produced with much higher reproducibility than printed cDNA arrays used in early times. The general method encompasses adding a label to the isolated mRNAs via enzymatic steps, hybridizing the labeled molecules to the array with immobilized DNA molecules, and then quantifying the label, which may be fluorescent on its own, or via binding of fluorescently labeled molecules allowing signal amplification. The strength of the signal at a certain localization representing a specific gene then is proportional to the amount of the corresponding mRNA in the original sample. A comparison between major microarray types, performed by the MicroArray Quality Control (MAQC) consortium, with participation by microarray platform providers, the US FDA National Center of Toxicological Research, and others, revealed high repeatability between and within platforms and high correlation between microarray results and other quantitative gene expression measurements (Guo et al. 2006).

Another now well-established method for transcriptomics is RNA sequencing based on next-generation sequencing (NGS). This technology allows much faster and cheaper sequencing of, for example, whole genomes than the sequencing technologies available before (Woollard et al. 2011). NGS is now widely applied for various omics investigations, e.g., in pharmacogenomics research to search, for mutations in cancer genomes, for evaluation of the epigenome concerning regulation

of gene expression, and for expression profiling. The latter is based on the fact that NGS can also count how many times the same sequence is available in a DNA pool.

A further major technique to measure RNA levels is quantitative polymerase chain reaction (Q-PCR), which allows sensitive quantification of one to several hundreds of known genes; Q-PCR may be used to validate expression profiling results or measure certain genes in many samples in a higher-throughput version.

Issues such as reproducibility of gene expression profiling techniques, standard practice for assays and analysis, relevance of the results to conventional endpoints, and robustness of statistical models on diverse data sets have been and are addressed by public consortia in addition to MAQC. Due to recommendations for technical aspects developed in such efforts, several expression profiling methods are now available for toxicogenomics investigations. These include highly standardized microarrays with reproducible performance and reasonable sensitivity, potentially more sensitive RNA sequencing protocols (Wang et al. 2014), and higher throughput versions of the latter like TempO-Seq (Bushel et al. 2018).

Proteomics

Proteomics, which encompasses identification and quantification of all proteins within a given proteome, is rather challenging and therefore higher throughput versions are being developed only now, which have not yet been widely applied in toxicology.

Reasons are (1) the much bigger complexity of the proteome compared to the genome due to the many possible protein modifications, (2) the wide dynamic range, (3) detection limits of the available technologies, (4) challenges with respect to characterization of membrane proteins, and (5) requirement for pre-fractionation (Ly and Wasinger 2011). Pre-fractionation can be performed with diverse methodologies, including various electrophoretic and chromatographic techniques. Then the separated proteins need to be extracted and identified with mass-spectrometric methods. Due to this rather elaborate workflow, proteomics analysis on a global scale were mostly used for specific mechanistic investigations. Recent developments include specific methods for certain questions, like investigation of the plasma protein patterns associated with different health conditions (Williams et al. 2019), or higher-throughput versions of immune-based methods with improved sensitivity (Greenwood et al. 2015). They may in the future also be used for toxicological questions.

Metabolomics

Metabolomics has the goal to comprehensively and quantitatively analyze all metabolites in a given sample, which could be cell or organ extract, or any biofluid. In toxicological studies, metabolomics may reveal affected metabolic pathways via

altered metabolite patterns which could either be causally involved in the toxic phenotype or represent the downstream effect of a toxic insult.

The large diversity in chemical and physical properties and the wide range of metabolite concentrations (nine orders of magnitude) pose great challenges to metabolomics methods. The two major technological platforms used are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS)-based approaches, the latter usually with prior chromatographic separation like liquid (LC) or gas (GC) chromatography. Although NMR is the more quantitative method, it is less sensitive than MS. Furthermore, since annotation of NMR peaks is rather time-consuming, NMR may be used to define peak patterns for different classes of toxicants yet is of less use for clarification of toxicity mechanism. Since MS-based methods allow construction of databases containing spectra for known metabolites, they can be employed in mechanistic toxicology.

Applications of Toxicogenomics in Toxicology

Fundamental assumptions of toxicogenomics are that all toxicological relevant effects are accompanied by gene expression changes and that similar toxicological mechanisms cause comparable expression changes, with potential exceptions being acute necrotic effects.

The idea to obtain insight into toxicological mechanisms from measurement of compound-induced gene expression has been around already before the appearance of expression profiling techniques, yet establishment of the latter permitted an analysis of these changes in their entirety, enabling to derive hypotheses about causative mechanisms.

A toxicogenomics study in general has three major components (Fig. 2): the biological model, the technological platform, and data analysis and interpretation, leading from application of a substance to a mechanistic hypothesis, to biomarker candidates or signatures, or to prediction of a potential toxicity. Both in vivo and in vitro models may serve as model, yet the studies need to be appropriately designed with respect to, e.g., time course and doses, and preferably should allow correlation of the expression profiles with conventional toxicological endpoints.

As outlined above, powerful technological platforms are nowadays available which enable examination of the influence of compound effects on essentially all genes in the corresponding target organs of the principle tox species. The major challenge then lies in analysis of the huge amounts of data being generated. Adequate methods must be applied for identification of a useful number of truly deregulated genes to derive a biological interpretation for the observed toxicological effects of a compound or compound class. Recommendations and best practices in this area have now been developed (Yauk et al. 2020).

Analysis of toxicogenomics data (Afshari et al. 2011; Chen et al. 2012) can broadly follow two major paths: (1) mechanistic analysis and (2) classification or prediction analysis. Biomarker genes, signatures or gene expression modules

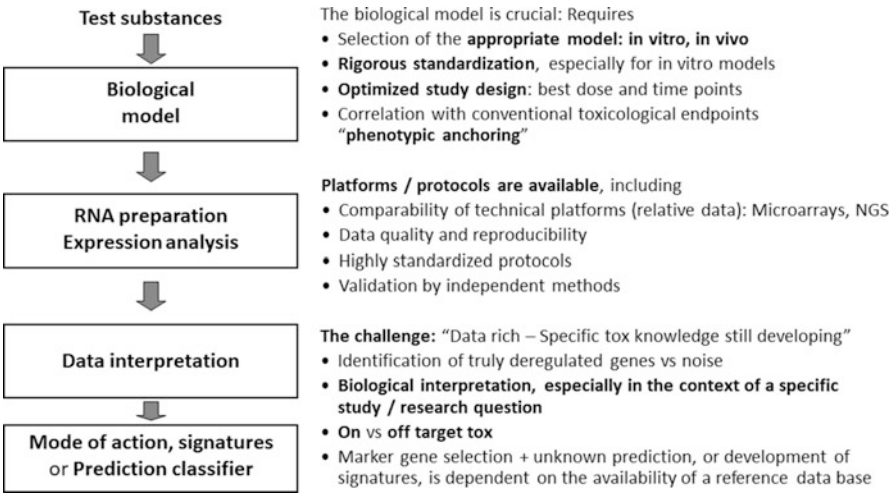

Fig. 2 Proper preparation and performance of the three major components of a toxicogenomic study is important to obtain good quality results

representing certain toxic effects may be derived from both approaches. A further extension of the mechanistic approach is benchmark dose (BMD) modeling of transcriptomic responses for derivation of provisional point of departure (POD) dose values for screening chemicals without published reference doses (RfDs) (Farmahin et al. 2017).

Mechanistic Toxicogenomics

Mechanistic toxicogenomics encompasses the assignment of functional categories to significantly deregulate genes in a biological model in the context of dose and time dependence and their relation to possible mechanisms of toxic action. From this analysis, mechanistic hypotheses may be derived or mechanistic similarities between different toxic agents may be uncovered. This approach can nowadays be seen as "State of the Art" in toxicogenomics analysis, as revealed, for example, by the many publications to characterize toxic compound effects with gene expression profiling. Examples can be found in the chemical and pharmaceutical area and include evaluation of liver and kidney toxicity (Suter et al. 2011), of nanoparticle-induced lung toxicity (Bourdon et al. 2013), or of short-term molecular responses to rodent carcinogens (Ellinger-Ziegelbauer et al. 2009).

The increased insight gained by such investigations can contribute to development of so-called adverse outcome pathways (AOP). AOPs describe the development of an adverse outcome after toxicant treatment with a series of events starting with the molecular initiating event (MIE) followed by several key events (KE) at the organelle, cellular, tissue, or even organisms' level, connected in a linear or branched

manner. This framework allows to organize information and knowledge on adverse events in a retrievable way and enables the development of simpler test batteries based on MIEs or KEs (Vinken et al. 2017).

Predictive Toxicogenomics and Signatures

Predictive toxicogenomics relies on a database of expression profiles from samples representing organs or cells following treatment with compounds of predefined toxic classes. Then marker genes are selected, and classifiers are calculated by statistical or other algorithms to allow classification of unknown samples with respect to potential induction of these toxicity classes. For quite some time, due to challenges including compilation of a sufficient number of expression profiles derived from studies with well-defined compounds, and appropriate bioinformatics methods, toxicogenomics was not widely used for prediction. Yet recently, two of the largest toxicogenomic databases were made public: TG-GATEs from the toxicogenomics project in Japan (Uehara et al. 2011) and DrugMatrix, originally generated by Iconix Pharmaceuticals (Ganter et al. 2005). Both databases contain gene expression profiles of the liver after up to 4 weeks treatment of male Sprague Dawley rats and of hepatocytes treated in vitro, with compounds representing liver toxicants and non-liver-toxic controls, including several genotoxic and nongenotoxic rat hepatocarcinogens. Due to their uniform experimental design, availability of extensive metadata for classical toxicological endpoints, and the inclusion of marketed drugs which should allow an assessment of whether gene-expression profiles measured in preclinical models can help predict human toxicity, they are of great value for various investigations, e.g., evaluation of prediction classifiers. Based on an overlap of 72 compounds in the 2 databases, Gusenleitner et al. (2014) built a classifier with DrugMatrix data for prediction of a chemical's carcinogenic potential and validated this classifier with the TG-GATEs dataset. This yielded an overall 80% accuracy, suggesting that prediction of certain endpoints based on gene expression data is possible with a well-annotated and sufficiently large data set of robust quality. Such results and the availability of more and more well-annotated omics data from studies with similar designs led to reconsiderations of the use of omics data in risk assessment. In the chemical area, short-term toxicogenomic studies for chemicals associated with no or only few safety data can be used for prioritization of chemicals for further testing, e.g., for 2-year cancer bioassays.

To develop transcriptomics signatures associated with nuclear receptor or other stress responses in the liver, Podtelezhnikov et al. (2020) profiled the transcriptional changes in rat liver following administration of 120 different compounds including drugs and chemicals known to be associated with drug-induced liver injury. Their iterative bioinformatic approach, starting with a small set of well characterized compounds, enabled them to derive optimized gene expression signatures for five canonical xenobiotic nuclear receptors (AHR, CAR, PXR, PPARα, and ER), three mediators of reactive metabolite-mediated stress responses (NRF2, NRF1, and P53), and one representing activation of the innate immune response in the liver. Although

not yet cast into a prediction classifier, further use of these signatures in collaborative settings is expected to aid in characterization of potential liver safety liabilities of drug candidates, especially when quantitative considerations are included in further testing. Overall these developments indicate that use of toxicogenomics for predictive approaches, or at least for characterization of potential chemical liabilities of chemicals is an option which will gain value through further use in chemical and pharmaceutical safety evaluation.

BMD Modeling

Benchmark dose (BMD) modeling (Farmahin et al. 2017) was developed as a pragmatic approach for a preliminary quantitative risk assessment for chemicals, for which only few data, and especially for which no longer term study data, are available. It builds on mechanistic interpretation of compound-induced gene expression changes and does not need databases of expression profiles. Here genes showing a dose-dependent expression response are identified with statistical methods in biological samples after exposure of the corresponding model to at least three doses of a compound for a certain duration. After fitting the profiles of these genes to standard statistical models, e.g., linear or polynomial, the best model is used to estimate the BMD for each gene at which its expression significantly deviates from that in control animals. Matching each gene with its corresponding functional category, e.g., gene ontology (GO) categories, and calculation of summary values per category, allows to derive a benchmark dose (BMD) level for the treatment-dependent change of a function. As for "no adverse effect levels" (NOAEL), uncertainty factors can then be applied to these BMDs, to finally determine a reference dose (RfD) for the most sensitive category/function. Transcriptional BMDs for gene expression data may be calculated with a free software called BMDExpress 2 (Phillips et al. 2019).

Thomas et al. who originally introduced this application have developed a framework for application of transcriptomic BMD modeling for both cancer- and non-cancer-related chemical risk assessment (Thomas et al. 2013). BMD modeling has now been employed and refined for chemical assessment by researchers at, e.g., EPA, NIEHS, Health Canada, for in vivo and in vitro evaluations (Ramaiahgari et al. 2019).

Weighted Gene Co-expression Network Analysis (WGCNA)

Weighted gene co-expression network analysis (WGCNA) is an approach to increase insight into the mode of action of toxicants inducing certain phenotypes, when only a limited number of treatments and thus expression profile data for the phenotypes of interest is available (Sutherland et al. 2018). When applied to the TG-GATEs rat liver data set, this analysis defined 415 modules of genes being co-expressed across all treatments, which can be organized in a visual representation of the transcriptome

(the "TXG-MAP"). Since the genes within a module represent certain functions, mechanisms or phenotypes of toxicity for certain compound treatments can be derived from the modules affected by these treatments. As an example, gene expression modules representing bile duct hyperplasia could be defined in rat liver, which were also perturbed in several human diseases with similar etiology.

The WGCNA modules originally described by Sutherland et al. (2018) were independently derived by a team at the University of Leiden within the TransQST consortium (http://transqst.org/news/the-txg-rmap-an-innovative-wgcna-tool). These modules are available in an open source application tool called TXG-MAPr (https://txg-mapr.eu/).

ICH(S1) Revision: Potential Use of Transcriptomic Signatures

Based on extensive analyses of chronic rat studies (Sistare et al. 2011) by pharmaceutical companies and health authorities (FDA, EMA, JPMA), the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) proposed to reconsider the ICH (S1) Guidance on Rodent Carcinogenicity Testing of Pharmaceuticals, and published a regulatory notice document in August 2013, which was updated in 2016 (Peden 2016). The goal of this proposed change is a more comprehensive and integrated approach to address the risk of human carcinogenicity of small molecule pharmaceuticals, and to define conditions under which 2-year rat carcinogenicity studies add value to that assessment. Specifically, this could mean that the 2-year rat carcinogenicity may be waived. During an evaluation period starting 2013, pharmaceutical companies were asked to submit Carcinogenicity Assessment Documents (CADs) in which they justify a waiver (or no waiver) to omit (or need to conduct) a 2-year rat carcinogenicity study for an investigational new drug, yet still perform and report the 2-year rat carcinogenicity study outcome. Based on weight of evidence (WoE) for a categorical assignment in the CAD (S. Table 1 in Peden (2016)), the following elements/studies and results thereof could be used: (1) pharmacology of intended drug target, (2) genotoxicity study results, (3) chronic (up to 6 months) study histological observations with emphasis on risk factors for carcinogenicity, (4) evidence of hormonal disturbance, (5) immune suppression, (6) metabolic profile, (7) transgenic mouse carcinogenicity study (not required, but may contribute), and (8) special studies and endpoints. To decide on a potential revision of ICH(S1), Drug Regulatory Authorities (DRAs) are currently evaluating the results of the 2-year rat carcinogenicity study with the categorical assignment proposed by the sponsor's in their CADs.

For the WoE element "Special Studies and Endpoints," transcriptomics data are one option. Along these lines, Rooney et al. (2018) derived transcriptomics signatures together with a scoring system from short-term rat studies for several molecular initiating events (MIEs) suggested to be associated with rodent hepatocarcinogenesis, linked with doses previously shown to induce (or not) liver cancer in the rat. They thereby could rank chemicals based on their ability to activate these

MIEs. It appears even possible to make a connection to cancerogenic versus non-cancerogenic doses. This approach can be expanded and refined with further datasets, which will be evaluated within the Emerging Systems Toxicology for the Assessment of Risk (eSTAR) Committee of the Health and Environmental Science Institute (HESI) organization.

Summary and Conclusion

After a hype in the beginning, as not unusual for new technologies, the application of "omics" in toxicology has had and still has its challenges, but after difficult times is now being reconsidered for use in regulatory risk assessment. It started with transcriptomics in 1999, when Spencer Farr suggested that all toxicological relevant effects are accompanied by gene expression changes, and that similar toxicological mechanisms cause comparable expression changes. This raised great hopes that toxicological outcomes might even be predictable from shorter term studies, which in the early years could not be confirmed. In the following years, researchers gained relevant experience with mostly mechanistic applications in the context of phenotypic anchoring. After several gene expression databases for compound classes causing certain toxicities in rodents were developed and became public, re-evaluation of predictive applications with renewed efforts enabled derivation of signatures which may enable classification of carcinogenic potential at least for rodent hepatocarcinogens including assessment of human relevance. These renewed efforts also led to development of alternative analyses strategies like WGCNA to improve mechanistic insight of toxicological phenotypes. Furthermore, benchmark dose modeling for transcriptomics data can deliver mechanistically anchored short-term in vivo study data for a first assessment of toxic levels for environmental chemicals associated with very few data.

Challenges still exist including further standardization of all elements of a toxicogenomics study, and development or refinements of gene expression signatures for potential qualification for a certain context of use. In addition, epigenomics data will need to be evaluated for use in toxicological assessments. Such investigations are most effectively pursued in precompetitive collaborative efforts which invite regulatory authorities as advisors. Examples for such collaborative efforts are working groups of the Predictive Safety Testing Consortium (PSTC) initiated by the FDA Critical Path Initiative, project committees belonging to the Health and Environmental Science Institute (HESI), and certain EU projects, e.g., the (now finished) IMI MARCAR project (biomarkers and molecular tumor classification for non-genotoxic carcinogenesis, http://www.imi-marcar.eu/). Furthermore, regulatory authorities, especially FDA and EMA, are evaluating (Goodsaid et al. 2010) and asking for submission of omics data and developed guidance documents for biomarker qualification to enable use of omics data in decision-making during drug development. Also, with respect to carcinogenicity assessment, researchers in the agrochemical and chemical area are considering approaches based on scientific understanding better suited to characterize the

carcinogenic potential of chemicals and to protect human health (Wolf et al. 2019). Therefore, it can be expected that these technologies will have significant impact on regulatory decisions in the future.

Cross-References

- ▶ Dose–Response Relationship and Extrapolation in Toxicology: Mechanistic and Statistical Considerations
- ▶ Toxicity Testing In Vitro: Regulatory Aspects

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Epidemiological Methods in Regulatory Toxicology

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Ulrich Ranft and Gregory A. Wellenius

Contents

U. Ranft (✉)
IUF – Leibniz Research Institute for Environmental Medicine, Düsseldorf, Germany
e-mail: ranft@uni-duesseldorf.de

G. A. Wellenius
Department of Environmental Health, Boston University School of Public Health, Boston, MA, USA
e-mail: wellenius@bu.edu

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Abstract

A major challenge in the field of toxicology is extrapolating the findings from in vitro and in vivo animal experiments to infer a causal effect of exposure on disease in people. Specifically, differences in species, exposure dose, route of administration, duration of follow-up, and co-exposures may lead to substantially different effects of exposures in animals or cells than in humans. Randomized trials or intervention studies in people provide an opportunity for assessing the health effects of exposures, but high costs and safety concerns often limit the types of exposures that can be evaluated with this approach. In the context of regulatory toxicology of potentially hazardous toxicants with little potential for benefit, safety and ethical concerns are paramount, leaving very few toxicants which can be ethically studied with randomized trials or intervention studies. Observational epidemiology provides a means to study the links between potentially harmful exposures and disease in people.

Keywords

Epidemiology · Cohort study · Case-control study · Confounder · Statistical power · Regression model · Random error

Basics

Definition

Epidemiology is often defined as the study of the distribution and determinants of diseases in people. By studying how specific exposures influence the distribution of diseases or physiologic variables, one can find evidence in support of or against the presence of an association between a given exposure and disease. Therefore, epidemiology offers an important alternative to human or animal experimentation in studying the etiology of disease and identifying the health effects of potentially harmful toxicants.

The distinguishing feature of epidemiologic studies compared to experimental studies is that in epidemiologic studies, exposure is determined by each individual (or their circumstances, environment, etc.) rather than by the investigator. Because each *individual's exposure is not assigned at random*, the major challenge of epidemiology is being able to interpret the results of epidemiologic studies as evidence in support of the presence or absence of causal effects.

Population

Toxicologic experiments typically compare the average or expected level of a given outcome in exposed versus unexposed cells or animals. Implicit in this approach is that the results are based on average effects across the population under study and may not apply to any single cell or animal. Similarly, the target of inference in epidemiologic studies is the population under study rather than the individual. For example, while we might find that exposure increases the average risk of a specific type of cancer, we generally cannot determine whether an individual that developed this cancer developed it as a result of the exposure under study. However, under certain assumptions, population effects can be used as *statements of probability* about the health risks of individuals. For instance, extending the prior example, we might be able to say that the risk of developing this cancer is higher in an exposed individual as compared to an unexposed individual.

Exposure and Effect

Epidemiologic studies are designed to quantify the association between a given exposure and a given outcome or health effect. However, an important aspect of epidemiologic study design is clearly defining what is meant by "exposure" and "effect." Figure 1 illustrates the *causal chain from exposure to outcome* as frequently

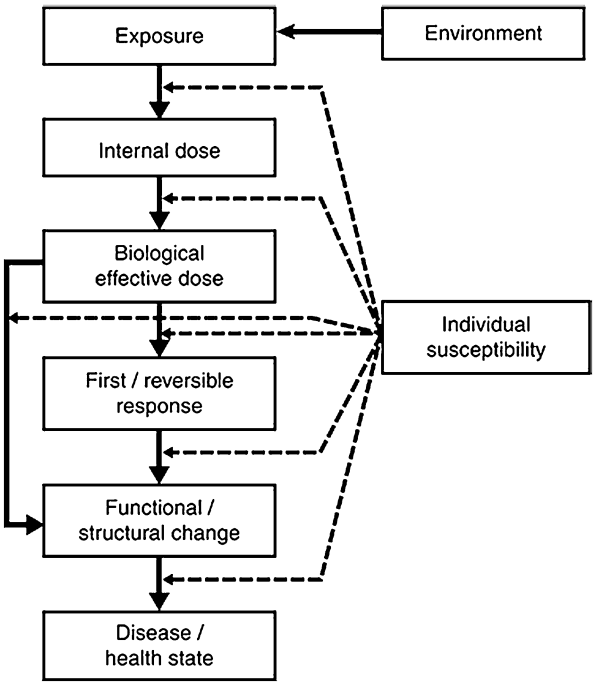

Fig. 1 Causal chain from exposure to disease

conceived. For example, in a study of the health effects of water disinfection by-products, we must clearly specify whether the exposure of interest is the concentrations of these by-products in the water supply (potentially combined with some estimates of individual water consumption rates), the concentration of some biomarker of internal dose, or some estimate of the biologically effective dose of the by-products. Similarly, we need to be very specific about the definition of the outcome, which can range from subtle shifts in physiological variables to incidence of overt disease. Due to its importance, estimating exposure has its own chapter. To reliably differentiate the health effects of several simultaneous exposures presents a particular challenge for statistical analysis and interpretation.

Association Versus Causation

As alluded to above, epidemiologic studies quantify the statistical association between a given exposure and disease. Two variables might be statistically associated because of the following: (1) uncontrolled confounding (as occurs when the two variables have a common cause), (2) selection bias (as occurs when participants are chosen in a way that is related to both exposure and outcome), (3) chance, and/or (4) one variable actually causes the other. A fundamental challenge of epidemiologic studies is to minimize the potential for confounding, selection bias, and chance so that the results of the study provide evidence in support of (or against) the presence of a causal effect of exposure on disease.

While much can be done during the study design and data analysis to minimize the potential for observing noncausal associations, in the end the interpretation of a statistical association as reflecting causality is based on judgment. An early formulation of guidelines for *inferring causation from observation* studies was provided by Sir Austin Bradford Hill (Table 1). Of the criteria shown, only temporality (exposure occurs before the outcome) is necessary for a causal relationship to exist. While the remaining Hill criteria are neither necessary nor sufficient to infer causation, they provide a useful framework for judging the strength of the evidence.

Table 1 Hill criteria for the evidence of a causal relationship

Strength of association
Consistency
Temporality
Biological plausibility
Dose-effect relationship
Coherence among epidemiologic studies
Specificity
Coherence between epidemiologic and laboratory findings
Analogy

Measures of Disease Occurrence and Association

Traditional epidemiologic studies aim to quantify the association between exposure and a dichotomous outcome, generally the presence or absence of a given disease. Disease occurrence is generally quantified using estimates of *prevalence*, *risk* (also called incidence proportion or cumulative incidence), and *incidence rate* (see Table 2, Fig. 2).

Common measures of association between exposure and a dichotomous outcome include the *risk ratio* (also called the cumulative incidence ratio), the *incidence rate ratio*, and the *odds ratio* (Table 2). When there is no association between exposure and outcome, the risk, rate, and odds of the disease will be the same in the exposed and unexposed groups, and the risk ratio, rate ratio, and odds ratio will all be equal to 1.

The nonspecific term *relative risk* risk, relative is often used to refer to any of these ratio measures. However, it is important to note that estimates of the risk ratio, rate ratio, and odds ratios have different mathematical properties and interpretations, and generally these terms cannot be used interchangeably. In particular, when exposure truly increases the risk of disease, estimates of the odds ratio will always be further from the null hypothesis of no association than the risk ratio, making the results appear more extreme.

Table 2 Common measures of disease occurrence and association

Measures of disease occurrence
Prevalence: the ratio of the number of existing cases of disease observed at a given point (*point prevalence*) or during a certain time period (*period prevalence*) to the size of the population under observation. A proportion ranging from 0 to 1
Risk, *incidence proportion*, *cumulative incidence*: The ratio of the number of new (incident) cases of disease within a given time period of observation to the number of people at risk of the disease at the start of observation. A proportion ranging from 0 to 1
Incidence rate: The number of new (incident) cases of disease within a given time period of observation divided by person-time at risk of the disease. Person-time is defined as the sum of the time spent under observation and at risk of disease by each member of the population. Incidence rates are not proportions and range from 0 to positive infinity
Measures of associations
*Risk ratio: R*isk of disease among the exposed divided by the risk of disease among the unexposed
Incidence rate ratio (*rate ratio*): Incidence rate of disease among the exposed divided by the incidence rate of disease among the unexposed
Odds ratio: Odds of disease among the exposed divided by the odds of disease among the unexposed where the odds of disease are defined as the probability of disease divided by (1 − the probability of disease)
Relative risk: a nonspecific term that can refer any ratio measure of association (i.e., risk ratio, rate ratio, or odds ratio)
Risk difference: Risk of disease among the exposed minus the risk of disease among the unexposed
Incidence rate difference: Incidence rate of disease among the exposed minus the incidence rate of disease among the unexposed

Fig. 2 Definition of epidemiologic measures

Contingency table

		Exposure: yes	Exposure: no	
Disease	yes	a	b	a+b
	no	c	d	c+d
	Total	a+c	b+d	n = a+b+c+d

Measure	Formula
Risk of exposed	a / (a+c)
Risk of non-exposed	b / (b+d)
Relative risk	a(b+d) / b(a+c)
Odds ratio	ad / bc
Risk difference	(ad – bc) / (a+c)(b+d)

Note:

When a, b, c and d are observed frequencies, then the formulae are estimates of the measures.

When a, b, c and d are probabilities (n=1), then the formulae are the true measures.

The association between exposure and a dichotomous outcome can also be quantified using difference measures, including the *risk difference* and *incidence rate difference* (Table 2). When there is no association between exposure and outcome, the risk (or incidence rate) in the exposed and unexposed groups will be the same and the risk (or incidence rate) difference will equal zero. Note that measures of relative risk assume that exposure acts to multiply the baseline risk (or rate or odds), while difference measures of association assume that exposures add to the baseline risk (or rate).

The above measures of disease occurrence and association are applicable to situations where the outcome is dichotomous. For simplicity, in the above discussion, we have implicitly also considered exposure as a dichotomous variable (i. e., comparing outcomes in those exposed vs. unexposed), but this is not necessary. The above measures of association can be generalized to situations where exposure is measured as a continuous variable.

A somewhat different set of metrics are used when studying the association between exposure and a continuous outcome such as blood pressure, heart rate, or

levels of a disease biomarker. In these settings, the expected values of the outcome (generally the arithmetic mean) among the exposed and unexposed subjects are compared.

Common Epidemiologic Study Designs

Descriptive and Analytical Epidemiology

Descriptive epidemiology is limited to the description of disease distribution using suitable measures, such as incidence rate, to enable comparisons between populations across space, time, or other contrasts. Descriptive studies are often carried out using routinely collected administrative or survey data. The results of descriptive epidemiologic studies are frequently used for public health planning or to generate new hypotheses about disease etiology. In contrast, analytical epidemiologic studies are used to test hypotheses about exposure-effect relationships. Study design and statistical evaluation focus on the initial assumptions and aim to make quantitative statements about associations which can be used to interpret the cause. The most important types of study in *analytical epidemiology* are briefly described below.

Cohort Study

A cohort study is an epidemiologic study design where participants that are initially free of a disease are followed for a specified period of time and monitored for new cases of (i.e., incident) disease. Exposures of interest and potential confounders are measured at study entry and, optimally, at multiple times throughout the follow-up period. In a *closed cohort study*, participants enter the study at baseline and remain under observation until they develop the disease of interest, die, or are otherwise lost to follow-up. Prominent examples of closed cohort studies include the Women's Health Initiative, the Cardiovascular Health Study, and the Nurses' Health Study. In an *open cohort study*, participants may enter and leave the study multiple times during the follow-up. Examples of open cohort studies include studies of all current members of a health insurance plan or all current residents of a state.

Cohort studies can be either *prospective* or *retrospective*. In a prospective study, data on exposure and confounders are obtained before the development of disease. In a retrospective study, historical data on exposure and confounders are assembled from existing data sources, often after disease has already occurred. Prospective cohort studies can require long follow-up of a large number of participants and are therefore frequently very expensive. Retrospective studies that make use of existing data can be very cost-effective but may be subject to additional potential biases.

Case–Control Study

A cohort study can be very inefficient if the disease of interest is rare. For example, suppose that we are interested in studying a disease that in a given population has an incident rate of 20 cases per 100,000 person-years. In this example, we could follow 100,000 people for 5 years and still only expect about 100 new cases of melanoma. Put another way, we would need to assess and follow for 5 years about 90,000 people that will not develop melanoma in that time frame.

Because the statistical power of a study is driven largely by the number of incident cases, in the above example, we could gain almost as much information if we were to assess only the 100 new cases of melanoma and a random sample of those participants that have not yet developed melanoma. Thus, the fundamental principle of a *case–control study* is that one can assess exposures and confounders in a *group of cases* (those with disease) and a (potentially small fraction) random sample of participants that have not yet developed the disease (*group of controls*). The strategy for selecting controls from the source population is very important for the interpretation and validity of the resulting estimate. For example, depending on the control sampling strategy, the odds ratio estimated from a case–control study may approximate either the incidence rate ratio or the risk ratio. The details of control selection strategies are beyond the scope of this chapter.

Sometimes a case–control study is nested within a larger cohort study such that the cases are those identified by the cohort and controls are sampled from among those participants in the cohort that have not yet developed the disease of interest. This is referred to as a *nested case–control study* and has the advantage that the source population giving rise to the cases is easily identified (i.e., the cohort participants). In other case–control studies, identification of the source population may be more challenging. For example, in a hospital-based case–control study, complicated referral patterns and changing catchment areas often make it difficult to clearly identify – and hence choose a random sample of – the source population that gave rise to the cases. Like cohort studies, case–control studies can be either prospective or retrospective in nature.

Cross-Sectional Study

In a *cross- sectional study*, participants are selected at random from a population of interest and then examined for risk factors and disease at the same time. Thus, the key feature of a cross-sectional study is that exposures and prevalent (existing) cases are assessed simultaneously. Because cross-sectional studies study *prevalent disease*, they generally provide estimates of prevalence ratios or prevalence odds ratios rather than risk ratios or incidence rate ratios. Moreover, because cross-sectional studies assess exposure and outcomes simultaneously, it is often not possible to clearly determine the temporal direction of the association. However, the duration of these studies is generally comparably short, making cross-sectional studies a cost-effective type of study appropriate for exploring certain hypotheses.

Panel Study

A *panel study* is a special type of cohort study where participants are followed longitudinally and both exposures and outcomes are assessed repeatedly over time within each participant. Panel studies are most appropriate for outcomes that vary over a relatively short time period such as blood pressure, weight, or levels of most serum biomarkers, but are not appropriate for most disease end points.

Ecological Study

All of the study designs presented above make use of data collected from individuals. In contrast, *ecological studies* quantify associations between a given exposure and outcome, but both the exposure and outcome are measured in *aggregate* rather than in individuals. Prominent examples of ecological studies include studies showing that average per capita meat consumption is associated with incidence rates of certain types of cancer compared across several countries. The key feature of an ecological study is that the unit of observation is the country (or county, neighborhood, school, etc.) rather than the individual. Because data are aggregated, there is considerable potential for uncontrolled confounding in ecological studies and incorrect conclusion (i.e., *ecological fallacy*). For instance, continuing the above example, smoking is also an important determinant of many cancers and may also be associated with higher meat intake in individuals. However, in an ecological study, controlling for average population smoking prevalence would not necessarily control for confounding by smoking at the individual level. Thus, ecological studies are most useful for offering initial evidence in favor of novel hypotheses.

Study Quality

Validity

The overall objective of an epidemiologic study is to obtain a valid and precise *estimate* of disease occurrence or of the association between exposure and disease. It is useful here to differentiate between internal and external validity. *Internal validity* {validity, internal} refers to whether the results of the epidemiologic study can be used to make inferences about the source population for that particular study. On the other hand, *external validity* {validity, external} refers to whether the results of the current study can be generalized to other populations.

Errors in epidemiologic estimates can be classified as either *systematic errors* {error, systematic} (*bias*) or *random errors* {error, random} (*chance*). Of note, while the potential for random error decreases with increasing sample size, the potential for systematic errors is independent of sample size. Moreover, the precision or amount of random error present in an estimate can be easily quantified with routine statistical

methods. In contrast, predicting the direction or magnitude of the bias induced by systematic errors is quite challenging and seldom done in practice.

Systematic errors can be further classified as due to confounding, selection bias, or information bias. Each of these sources of systematic bias is discussed in more detail below.

Confounding

In contrast to experimental research, confounding is a major threat to validity in epidemiologic studies. *Confounding* {bias, confounding} can occur when one or more factors exist in the study population which are associated with both the outcome and the exposure, but are not caused by either the exposure or the outcome (Fig. 3). A known risk factor is a potential confounder and, if associated with the exposure in the study population, will become a *confounder*. Importantly, confounding can bias the health effect estimates either towards or away from the null hypothesis of no association.

The potential for confounding can be minimized through appropriate study design. For example, if sex is an important confounder (because it is associated with both exposure and disease but not caused by either), a study restricted to men only or women only would not be susceptible to confounding by sex. More commonly, analytic methods are used to reduce the potential for confounding. For instance, continuing the above example, if sex is an important confounder, we can stratify the analyses on sex, that is, consider the association between exposure and disease conditional on sex. More generally, one can use regression models to quantify the association between exposure and disease conditional on (or adjusting for) a number of potential confounders.

Selection Bias

Selection bias {bias, selection} generally arises from the manner in which participants were selected for the study. A typical example of selection bias is the so-called

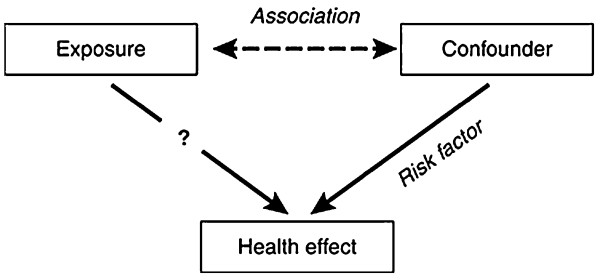

Fig. 3 Confounding: relationship between exposure, health effect, and confounder

nonresponder bias {bias, non-responder} in which people that agree versus those who do not agree to participate in a study differ in terms of both exposure and their risk of the outcome. If the factors which potentially influence the selection are known and therefore measured, the selection bias can be controlled in the statistical analysis (see confounding).

Selection bias may also occur in the context of case–scontrol studies (see below) where the controls are meant to represent the distribution of exposure among the source population from which the cases arose. *Selection bias in a case–control study* occurs when the controls are sampled in such a way that the exposure distribution among the controls does not estimate the exposure distribution in the source population.

Another common source of selection bias arises in the setting of missing data or when participants are *lost to follow-up*. If the risk of a subject missing data is related to both exposure and outcome, the missingness is said to be informative. Ignoring informative missing data can lead to selection bias. Similarly, loss to follow-up in a cohort study (see below) leads to missing data in some study participants. If the risk of being lost to follow-up is associated with both the exposure and outcome, selection bias may result.

Information Bias {Bias, Information}

Exposures and outcomes in epidemiologic studies are always measured with error. Measurement error of dichotomous or categorical variables is often referred to as *misclassification*. Misclassification which depends on another variable is termed differential misclassification. Misclassification that does not depend on other variables is termed *non-differential misclassification*.

For example, differential misclassification of the exposure would occur if an exposure were measured with more error among those with the outcome of interest as compared to those not experiencing the outcome of interest. *Recall bias* {bias, recall} in the context of case–control studies is a well-known example of differential exposure misclassification; the health effect estimates are biased because those diagnosed with a specific disease (the cases) may recall their past exposures better than the controls without the disease. Differential misclassification can bias the estimated associations between exposure and disease either towards or away from the null hypothesis of no effect.

The impact of non-differential misclassification is sometimes predictable. Specifically, non-differential misclassification of a dichotomous exposure is expected to bias health effect estimates towards the null. This observation is often cited as a reason why one should not be overly concerned with measurement error of the exposure. However, non-differential misclassification of a categorical exposure with more than two categories, or a continuous exposure, can lead to bias either towards or away from the null, because in these cases the impact on study validity will depend on the pattern of classification and the measure of association.

Effect Measure Modification and Interactions

Effect measure modification {modification, effect measure} (frequently simply referred to as *effect modification* or *interaction*) is present when the association between exposure and disease differs across levels of a third variable (the modifier). For example, the relative risk between exposure to asbestos and lung cancer is known to be greater among smokers than among nonsmokers. In this example, it would be tempting to conclude that smokers are more susceptible to the effects of this exposure. However, for any given exposure-disease relationship, the presence of effect measure modification will depend on the measure of association being used, hence the term effect *measure* modification. For example, for a truly harmful exposure and in the absence of any other biases, the absence of effect modification when considering the risk ratio guarantees that effect modification will be present when one instead considers the risk difference. Effect modification is equivalent to the concept of *statistical interaction* {interaction, statistical} and represents a departure from a multiplicative or additive model, depending on the measure of association being modeled. Thus, it is entirely possible that effect measure modification will exist only in multiplicative models, only in additive models, or in both. Thus, the presence of statistical interaction or effect measure modification must be distinguished from the concept of biological interaction.

Students of epidemiology often have trouble distinguishing the concepts of confounding and effect modification. To clarify, effect modification is present when the strength of the association differs (aside from random variation) across strata of the potential modifier. On the other hand, confounding represents a mixing of the effect of the exposure with effects of other factors (confounders) on the outcome. Furthermore, confounders, by definition, are associated with exposure and outcome, and need to be controlled for in analyses. In contrast, an effect modifier need not be associated with either exposure or disease and need not to be adjusted for in analyses. A given factor potentially can be a confounder, an effect modifier, both, or neither.

Random Error and Precision

The precision of a health effect estimate (i.e., magnitude of the random error) in an epidemiologic study can be quantified and will depend on the *sample size*. This means the *precision* of study results can generally be improved by increasing the sample size of the study. In *study planning*, this is used to determine the sample size needed to answer a specific research question. Furthermore, it leads to the concept of *statistical power*, i.e., the ability of a study to demonstrate an association of a given magnitude between exposure and outcome, if such an association actually exists.

One can often increase the precision of estimates from an epidemiologic study by improving study efficiency. For example, for the same number of study participants, some epidemiologic study designs may be much more efficient than others. For instance, a case–control study with 1,000 cases and 1,000 control subjects is

Table 3 Principles and elements of "Good Epidemiological Practice"

Accordance with ethical principles
Formulation of explicit and operationalizable research questions
Detailed and binding study protocol
Well-documented biological sample banks
Quality assurance
Data management and documentation
Analysis
Observance of applicable data protection regulations
Legally binding agreements between all stakeholders (researchers, sponsors, collaborators)
Publication and interpretation of results

expected to be more informative than a case–control study with 500 cases and 1,500 controls or a cohort study that follows 2,000 subjects, 100 of which develop the outcome of interest. In the above example, study efficiency can be assessed as the amount of information per subject with some study designs yielded more or less information per subject. However, depending on the resource constraints, one may wish to optimize the amount of information per research dollar spent rather than per subject. For example, if recruiting cases for a case–control study is much harder (i.e., more expensive) than recruiting control subjects, a study with fewer cases and relatively more control subjects may be the most cost-effective.

Study Planning

A high *quality standard* in an epidemiologic study, usually conducted as a multidisciplinary cooperation, is only achieved when a *study plan* is developed at the start of each study which follows the generally recognized *Good Epidemiological Practice* (*GEP*) guidelines. In the international technical literature, guidelines have been developed which have found broad recognition and which contribute significantly to quality assurance if they are carefully followed. Some of the important principles and elements of the GEP are listed in Table 3.

Statistical Analysis

Estimation, Confidence Intervals, and Testing

Statistical procedures and statistical inference are material parts of epidemiologic methodology. Since statistics is accorded its own chapter (see ▶ Chap. 21, "Statistical Evaluation Methods in Toxicology"), this section will merely address three important viewpoints, and in the following section, an introductory explanation of the regression models will be given.

The estimation of measures of association, such as a risk difference or odds ratio, is subject to random error. Virtually always, quantitative study results on the exposure-outcome association are given as a combination of an estimate of the strength of the association (*point estimate*) and an estimate of the uncertainty or precision associated with the point estimate. Depending on the target audience and the statistic being used, the uncertainty may be expressed using the *standard error* of the point estimate or the 95% *confidence interval* around the point estimate. Since the confidence interval provides information on both the magnitude and the probability of a potential error, it is often the preferred method for quantifying estimate uncertainty in epidemiologic studies.

An important question in epidemiology relates to the significance of *testing hypotheses* {testing, hypotheses}. The purpose of testing a hypothesis is to come to a decision as whether to accept or to reject a hypothesis on the basis of the results of a trial which was carried out for this purpose. This methodological starting point of the statistical test theory does not, strictly speaking, apply to the problem of an epidemiologic study, which is to quantify the exposure-outcome relationship rather than to make a decision about whether or not the relationship exists. However, it is quite sensible to take the *p- value* from the hypothesis testing, which is a continuous evidence measure of the compatibility of a hypothesis with the data observed, but the p-value should not merely be used with a predetermined threshold value such as a 5% threshold to classify the results of the study as "significant" or "not significant."

Another point which is often hotly debated is *multiple testing* {testing, multiple} or more specifically the simultaneous investigation of several relationships in a study. Whether or not using procedures for simultaneous testing is recommended depends on whether the problem actually requires the simultaneous testing of a whole list of relationships (exposure to several end points of effect) or whether the various exposure-effect combinations are based on independent problems and, for example, are only being investigated together in one study because of practicability. In this latter case, no multiple testing is carried out. If multiple testing with a few simultaneous hypotheses is necessary, simple methods such as the *Bonferroni* method are available. For the analysis of more extensive sets of related hypotheses, modern but more complex procedures have to be used.

Regression Models

As discussed above, epidemiologic studies are susceptible to confounding, and potential confounders need to be considered either in the design of the study (through restriction) or in the analysis of the resulting data (through stratification or statistical adjustment). In very simple cases involving only a small number of key confounders, tabular analyses with simple hand calculations can be used to obtain valid point estimates and confidence intervals. However, as the number of potential confounders increases, tabular analyses become impractical and statistically inefficient and regression models are preferred. Choosing the most appropriate *statistical model* {model, statistical} is complex and requires advanced understanding of the

statistical issues, as well as a clear understanding of the hypotheses to be tested and the structure of all the relevant cause-effect relationships. Thus, *interdisciplinary cooperation* between epidemiologists, statisticians, and experts of all the affected specialties to create the model and evaluate and interpret the results is highly recommended.

Cross-References

► Statistical Evaluation Methods in Toxicology

Recommended Reading

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Early Clinical Trials

20

Simon Jäger and Matthias Schwab

Contents

Abstract

Studies in volunteers are most important in the research and development of a medicinal product or, as defined by the US Food and Drug Administration (FDA), of a drug. The initiation of clinical drug trials in humans requires comprehensive

S. Jäger
Department of Clinical Pharmacology, University Hospital Tübingen, Tübingen, Germany
e-mail: Simon.Jaeger@ikp-stuttgart.de

M. Schwab (✉)
Department of Clinical Pharmacology, University Hospital Tübingen, Tübingen, Germany

Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany
e-mail: matthias.schwab@ikp-stuttgart.de

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and substantial information about the investigational medical product, particularly toxicology, in order to exclude potential risks for subjects/patients with the greatest probability. Early clinical trials aim to provide first evidence of safety of the investigational medical product. Regulatory and ethical aspects must be taken into account as closely as possible.

Keywords

First-in-human · Phase I · Early clinical trials · Investigational medical product · Risk mitigation · Dose escalation · Multiple ascending dose

Introduction

The transition from nonclinical to early clinical trials is a crucial phase in the development of new investigational products (IMP). Calculation of the starting dose, dose escalation, and the definition of the maximum dose requires adequate nonclinical (or preclinical) data and specific consideration of safety pharmacology, reproductive or genetic toxicity, and carcinogenetic potential of the IMP.

The subsequent clinical trials in humans are conducted in a series of *phases* (Phase I to phase IV) as depicted in Fig. 1.

The following chapter focusses on early clinical trials in humans. Here, most commonly cohorts of healthy volunteers (HV) are enrolled. Selecting HV allows for controlling important confounders that may change the pharmacokinetics and

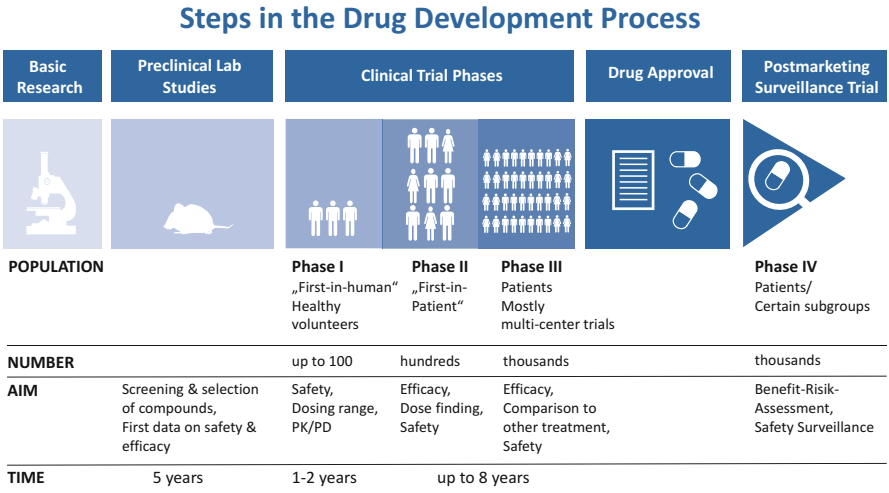

Fig. 1 Steps in the drug development process with focus on clinical trial phases. Clinical trials follow a rigorous series from early clinical trials (phase I/II studies) to late-stage, large scale, phase III studies. *PK* pharmacokinetics, *PD* pharmacodynamics

tolerability of the IMP, most importantly age, sex, or concomitant medication. Enrolment of HV also increases study accrual rates for the early dose-escalation studies.

Not least because study participants do not derive any health benefit, highest precautions to avoid any hazard for the participating HV must be guaranteed. Moreover, adequate compensation for time expenditure, discomfort, and inconvenience has to be ensured. All these aspects have to be approved by the institutional review board/ethics committee before a study can start.

Despite the advantages of a homogenous study population, IMPs with potential desirable toxic effects, particularly in the case of anticancer drugs, cannot be investigated in HV due to obvious ethical reasons. In these cases, phase I studies are conducted in the target population (i.e., actual patients). Yet, the employment of subtherapeutic doses in patients who suffer from the target disease raises legitimate ethical concerns on their own. Later studies (e.g., phase II), where clinical efficacy is a primary endpoint, may be more appropriate for patients (Karakunnel et al. 2018).

Phase I Studies

In phase I studies – also called human pharmacology studies – a new IMP is evaluated for the first time in humans (Fig. 1). Phase I studies comprise several types of studies. First-in-human (FIH) studies are also called single ascending dose (SAD) studies and are randomized, placebo-controlled studies. Here, a single dose is given and escalated over 6–10 dose groups of around eight healthy volunteers. The pharmacokinetic profile, safety, and tolerability are key aspects. Usually, a multiple ascending dose (MAD) study will follow to evaluate these parameters when multiple doses of the IMP are given over a longer period of time. If a single or double blind study design is employed, preliminary data on pharmacodynamics can also be obtained.

IMPs that have shown effects on the cardiac conduction system in preclinical studies have to be evaluated in a so-called thorough-QT-study (TQT study) to rule out a significant QT-prolongation in humans. Such alterations noted in electrocardiograms (ECGs) are associated with fatal arrhythmias. TQT studies are complex, placebo-controlled, double-blind trials with around 50 healthy individuals.

Other types of phase I studies, performed in an open design, include bioavailability (how much and how fast does the IMP enter the systemic circulation) and bioequivalence studies (differences in formulation, e.g., powder versus tablet).

In addition, food-effect studies and ADME (**A**bsorption, **D**istribution, **M**etabolism, **E**xcretion) studies are important to further characterize the drug and discover relevant metabolites. Information on excretion in urine and feces is collected in so-called “mass balance” studies. Guidelines by EMA or FDA describe how drug-drug interactions (DDI, e.g., interaction of the IMP with expected comedication) have to be investigated in phase I study programs. DDI studies evaluate clinically relevant drug-metabolizing enzymes (e.g., cytochrome [CYP] P450 enzymes) and drug transporters (e.g., the efflux-transporter P-glycoprotein) and their impact on drug levels. As an example, comedication with contraceptive pills should be investigated in an IMP whose target-population includes women of child-bearing potential.

Dedicated pharmacokinetic analysis in hepatic or renal impairment is not possible in healthy volunteers. In this regard, phase I/IIa studies have to be set up in appropriate cohorts, in collaboration with academic medical centers or university hospitals. Crossover designs to show intraindividual effects are employed in bioavailability, food-effect, and TQT and DDI studies, while parallel-group designs are used in SAD and MAD studies.

Another specific study type is a so-called microdosing study where subpharmacological doses are administered to a low number of humans for a short period of time. These studies are carried out in advance of the traditional dose escalation, safety, and tolerance studies. Hereby, HV receive an IMP to allow for an investigation of its pharmacokinetic properties while maintaining an extremely low risk of toxicity (Marchetti und Schellens 2007).

Safety and Toxicity in Nononcologic Phase I Trials

According to several analyses, phase I trials in HV with nononcology drugs are perceived to be safe. Emanuel et al. performed a meta-analysis of 394 Phase I studies conducted between 2004 and 2011 by a leading research-based pharmaceutical company. Of 11,028 HV, 36.3% experienced no adverse events and 63.7% experienced adverse events. Yet, 85% of adverse events were mild, and only 34 (0.31%) serious adverse events occurred, with no life-threatening events or deaths (Emanuel et al. 2015). Another systematic analysis included 25 phase I SAD and/or MAD studies and could not identify safety concerns involving laboratory parameters, ECGs, SAEs, or deaths (Young et al. 2017). Furthermore, a survey of phase I studies in Germany reported 1,250 subjects (950 active, 300 placebo), and 952 AEs occurred while 88.2% (840/952) of AEs were mild and 11.3% (108/952) were moderate. Only 0.4% (5/1,250) of subjects had active drug- or procedure-related serious AEs (Jung et al. 2020). These data are supported by previous analysis of AEs in phase I studies ($n = 142$) in Germany, indicating that AEs in phase I trials are common, but usually of mild or moderate degree (Lutfullin et al. 2005).

Nonetheless, nononcology early phase studies are not exempt of serious complications. In March 2006, a novel agonist antibody (TGN1412) directed at CD28 was studied in the first phase I trial (6 verum, 2 placebo). The substance has been well tolerated by nonhuman primate species. Despite being administered at a supposedly subclinical dose, the compound led to sudden and rapid release of proinflammatory cytokines, causing catastrophic systemic organ failure in six subjects after 12–16 h of infusion (Suntharalingam et al. 2006; Brøsen et al. 2017).

Although all six HV in the TGN1412 study survived after intensive care treatment, in a phase I study conducted 10 years later with the fatty acid amide hydrolase (FAAH) inhibitor BIA10-2474, there was one fatality due to neurologic complications. FAAH breaks down neurotransmitters known as endocannabinoids, but the symptoms have been speculated to be unknown off-target

effects of BIA10-2474 (Butler und Callaway 2016). These tragic occurrences are reminders that outmost care and meticulous attention to results from nonclinical trials are essential for a safe conduct of first-in-human studies.

Benefit-Risk Assessments

The assessment of risk of a phase I trial is a unique challenge. In the "Guideline on strategies to identify and mitigate risks for FIH and early clinical trials with IMPs," the European Medicines Agency (EMA) mandates the identification of specific risks (European Medicines Agency 2017). The novelty and extent of knowledge of the supposed mode of action is particularly important. If an IMP may interfere with multiple signaling pathways or biological cascades (e.g., hemostasis), a greater risk for severe adverse events from off-target effects has to be anticipated. Similarly, a lack/scarcity of knowledge about the structure, tissue distribution, regulation, or biological function of the target increases risks. Additionally, the availability of relevant animal models and their extrapolability factor into the risk assessment. In line with the Declaration of Helsinki, the Good Clinical Practice Regulation and national medicine acts, a concise benefit-risk assessment, is an essential part of the study application at the competent authorities and ethic committees. The sponsor has to detail the substance-related risks in the investigator brochure and the study-specific risks in the study protocol.

Phase II Studies

Phase II studies enroll actual patients and are usually conducted in medical centers with expertise in the particular target disease. Most commonly, they are managed by clinical research organizations (CRO) that specialize in the logistics of study conduct. Phase II studies are also called therapeutic explorative studies (Fig. 1). Their primary aims are efficacy assessment and dose finding, as well as safety and pharmacokinetics. A distinction between IIa and IIb studies, where IIa are proof-of-concept studies and phase IIb are dose-finding studies, can be made. Yet, studies incorporating both study questions in one design are common (IIa/b studies). There is also methodological discussion around phase II study designs. For example, designs that aim at an early validation of predictive biomarkers have been proposed. This would allow for timely decision if the drug should be developed for an unselected patient population or for a biomarker-defined subset of patients only (Mandrekar et al. 2013).

Phase III Studies

Therapeutic conformative trials, enrolling around 1,000 patients to show efficacy and safety, are referred to as phase III or "pivotal studies" (Fig. 1). Only compounds that have shown effects in phase II will enter the costly and logistically challenging

phase III. These studies are designed to establish superiority to placebo or non-inferiority to a comparator in large cohorts. This is prerequisite for a promising initial marketing authorization at the FDA or EMA. A so-called clinical development plan will have to specify how many phase III trials should be planned and conducted (Singh 2018). The choice of endpoints, comparator, number of subjects, study site, and location (e.g., EU, USA, and developing countries) will have a significant impact on the products' chances for marketing authorization. In 2009, Munos published an analysis of around 1200 drugs that have been approved by the FDA, since 1950, and pointed out that the probability of a new therapeutic molecule to enter such late phases is estimated to be only about 21.5 % or even 11.5 % only (Munos 2009).

Phase IV Studies (Postapproval Studies)

Phase IV studies are conducted after marketing authorization. Hereby, information on the drug's effectiveness in various populations is gathered, and any adverse effects associated with long-term use can be investigated. Serious adverse effects, if rare, may emerge with large-scale use only, and continuous safety reassessment by regulatory authorities may, in some specific cases, necessitate the restriction of the use or the withdrawal of the drug. Furthermore, a drug may have been approved based on surrogate end points likely to predict clinical outcomes. After such an approval, a phase IV study can be conducted to demonstrate effects on clinical end points (European Medicines Agency 2019). In addition, phase IV studies are also suitable to refine dosing recommendations or assess outcomes relevant for health technology assessments.

Early Clinical Development of Vaccines

Selected aspects and general principles of the clinical development of adjuvant or nonadjuvant vaccines will be reviewed in this paragraph, largely referring to the guideline on clinical evaluation of vaccines, issued by the EMA (European Medicines Agency 2018).

Before efficacy studies can be conducted, a careful characterization of the immune response elicited by the vaccine is necessary. This includes, for example, information on the functionality and nature of the induced antibody, the induction of memory cells, quantifying T cells specific for the vaccine antigen, etc. It has to be noted that the parameters which are of the highest significance for protection will not be known at this stage. Comparative immunogenicity trials commonly employ the seroconversion rate (i.e., rise in antibody concentration pre- and postvaccination) as a primary end point. Percentages of antigen-specific T cells, geometric means for antibody concentrations, and the postvaccination sero-positivity are accepted secondary end points.

In vaccine development, data from in vivo nonclinical studies are usually not helpful to determine the human dose. As it is not known what can be considered a protective immune response, the dose at which there is no further increment in immune response should be investigated. The differential effects in immune response to conjugated antigens and conjugative proteins have to be characterized as well.

An adjuvant formulation may allow for a reduction of the amount of the antigenic components of a vaccine. Yet, the employment of adjuvants (designed to augment the immune reaction) has to be corroborated by an adequate comparison between adjuvant versus nonadjuvant formulations. In inactivated vaccines, aluminum salts have been used for decades as adjuvants. Yet, although injection-site reactions are more common with vaccines containing aluminum, serious long-term hazards like increased rates of atopic diseases have not been confirmed by independent studies (Jefferson et al. 2004; Willhite et al. 2014).

Also, the safety of vaccines containing the organic mercury compound thiomersal has been a long-standing public concern, yet available data from large and well-conducted epidemiologic studies show no association with thiomersal-containing vaccines and neurodevelopmental defects (autism, speech disorders). It is used as an antimicrobial in early stages of manufacturing or as a preservative. Yet, reducing environmental exposure to mercury is a global concern. Therefore, reduction or removal of thiomersal has been achieved, and new vaccines without thiomersal are now available (European Medicines Agency 2004).

Regarding vaccines against SARS-CoV-2, multiple vaccine types, such as nucleic acid-, viral vector-, virus-, and protein subunits, are under investigations or have been approved. The attractiveness of RNA-based vaccines is the advantage of an easier and faster manufacturing in large quantities. Nevertheless, phases of vaccine development are subject to the same regulatory requirements comprising phase I to III trials (Mellet and Pepper 2021).

Important Regulatory Aspects

Studies in humans are subject to legal regulations (national and supranational, e.g., the European Union) which include so-called Good Clinical Practice (GCP) directives and professional regulations (Declaration of Helsinki, evaluation by independent review boards (IRBs)). In addition, institutional regulations (e.g., standard operating procedures) usually apply also.

Since its inception in 1990, The International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has become a pivotal organization, bringing together regulatory authorities and pharmaceutical industry (ICH 2021). It discusses scientific and technical aspects of pharmaceuticals and develops numerous guidelines, such as the ICH-GCP guideline (European Medicines Agency 2016; European Commission 2005). Those guidelines were also converted into German law as the "GCP regulation" (Federal Ministry of Justice and Consumer Protection 2012).

GCP is a standard for the design, conduct, performance, monitoring, auditing, recording, analysis, and reporting of clinical studies that provides assurance that the data and reported results are credible and accurate and that the rights, integrity, and confidentiality of study subjects are protected. It includes ethical and scientific quality standards for designing, conducting, recording, and reporting studies that involve participation of human subjects to ensure that the rights, safety, and well-being of the study subjects are protected. It is also to ensure the credibility of clinical study data.

Study Responsibilities and Roles

The *sponsor* is an individual, company, institution, or organization which takes responsibility for the initiation, management, conduct and financing of a clinical study. The *investigator* is a person, usually a physician, responsible for the conduct of the clinical trial at a study site. The investigator delegates appropriately qualified persons (e.g., physician, study nurses) to trial-related duties.

Trial Databases

According to the GCP-V regulation, all trials conducted in the European Union have to be entered into the *EudraCT* (European Union Drug Regulating Authorities Clinical Trials). EudraCT is the European Clinical Trials Database of all clinical trials commencing in the European Union from 1 May 2004 onward (https://www.clinicaltrialsregister.eu/). Internationally, the ClinicalTrials.gov registry (https://clinicaltrials.gov/), maintained by the US National Library of Medicine, is the largest database of privately and publicly funded clinical studies which are conducted worldwide. The "NCT numbers" are therefore the most common identifier found in scientific publications. Over the years, the entries have evolved to complete listings of study/start dates, inclusion and exclusion criteria, primary and secondary end points, and contact information. Recently, links to complete study protocols and statistical analysis plans as well as quick web links to pertinent publications have been added, further increasing transparency and usefulness of this free online resource.

Institutional Review Board (IRB)/Independent Ethics Committee (IEC)

An) *Independent Ethics Committee* (IEC) is an independent body (a review board or a committee, institutional, regional, national, or supranational), constituted of medical professionals and nonmedical members, whose responsibility is to ensure the protection of the rights, safety, and well-being of human subjects involved in a trial. IRB/IEC review and approve/provide a favorable opinion on the trial protocol, the

suitability of the investigator(s), facilities, and the methods and material to be used in obtaining and documenting informed *consent* of the trial subjects. The legal status, composition, function, operations, and regulatory requirements pertaining to IRB/IECs may differ among countries but should allow the IEC to act in agreement with GCP as described in the ICH-GCP Guideline (European Medicines Agency 2016). IRB/IEC also assure that the proposed clinical trials are conducted in accordance with the ethical principles that have their origin in the *Declaration of Helsinki.* The World Medical Association (WMA) has developed the Declaration of Helsinki as a statement of ethical principles for medical research involving human subjects, including research on identifiable human material and data (64th WMA General Assembly 2013).

Important Documents

The most important documents to be submitted to and reviewed by the IEC include:

The Study Protocol

The protocol is a document that describes the objective(s), design, methodology, statistical considerations, and organization of a trial. The protocol usually also gives the background and rationale for the trial, but these could be provided in other protocol referenced documents. Throughout the ICH-GCP Guideline, the term protocol refers to protocol and protocol amendments (European Medicines Agency 2016). The protocol also described the investigator's and sponsor's responsibilities. This is particularly important with the documentation, reporting, and assessment of adverse events.

The Informed Consent Form

The informed consent is a process by which a subject voluntarily confirms his or her willingness to participate in a particular trial, after having been informed of all aspects of the trial that are relevant to the subject's decision to participate. Informed consent is documented by means of a written, signed, and dated informed consent form (European Medicines Agency 2016).

The Investigator's Brochure (IB)

The IB is a compilation of the clinical and nonclinical data on the investigational product(s) that is relevant to the study of the product(s) in human subjects. Its purpose is to provide the investigators and others involved in the trial with the information to facilitate their understanding of the rationale for, and their compliance

with, many key features of the protocol, such as the dose, dose frequency/interval, methods of administration, and safety monitoring procedures. The IB also provides insight to support the clinical management of the study subjects during the course of the clinical trial (European Medicines Agency 2016). Obviously, the IB needs to include all the current knowledge about the investigational product(s).

Testing of Other Substances

To assess the tolerability of working materials and chemicals, controlled studies are occasionally performed in volunteers. Examples are studies of ozone effects on lung function or the study of the neuropsychological effects of low doses of solvents. Special rooms and equipment allow the inhalation of defined substance concentrations. Such studies are important in toxicology as for the revaluation of limit values for humans. The formalities required to perform such studies are usually similar to those of phase I clinical trials.

Cross-References

- ▶ Ethical Issues in Science: Focus on Regulatory Toxicology
- ▶ Examination of Acute and Repeated-Dose Toxicity
- ▶ Purpose and Methods of Risk Management in Regulatory Toxicology
- ▶ Toxicological Risk Assessment in Different Jurisdictions

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Statistical Evaluation Methods in Toxicology

21

Ludwig A. Hothorn

Contents

Abstract

What is specific to the statistics in toxicology, and why not just use textbook statistics? The reason is the aim of regulatory toxicology: "be confident in negative results." By toxicological studies, one would like to prove the harmlessness of new drugs. By means of the so-called proof of safety approach, the false-negative error rate (consumer's risk) is directly controlled. Unfortunately, in most of the statistical textbooks and publications, the alternative proof of the efficacy of new drugs with the direct control of the false-positive error rate is used, denoted in toxicology as proof of hazard. Therefore, in this chapter, the basics of the falsification principle are presented simplistically. The commonly used proof of hazard approach is discussed hereinafter, focusing on testing a dose-related trend. Finally, the proof of safety methods for selected study types is explained by means of examples.

L. A. Hothorn (✉)
Leibniz University Hannover (retired), Hannover, Germany
e-mail: ludwig@hothorn.de

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Statistics · Proof of hazard · Proof of safety · Significance tests

Introduction

In regulatory toxicology, statistical significance tests are used as standard for data evaluation. The difference between methods that directly control the more important false-negative error rates (proof of safety) and the common methods that directly control the false-positive rates (proof of hazard) is highlighted. Dunnett and Williams tests are recommended for the usual comparison of several dose groups versus the negative control, with simultaneous confidence intervals recommended for presentation and interpretation.

The Falsification Principle

Most of the tests used in biostatistics base on Popper's falsification principle, briefly "An effect can never be proved directly, only in that the probability of its opposite is very low." This very small probability is the p value of a test. It is a probability between 0% and 100%, where only very small levels, e.g., 0.01%, argue against the null hypothesis. As an arbitrary limit, 5% has been established; alternatively, for common tumors, a level of 1% was proposed as relevance criteria. Commonly, the decision for either the null hypothesis (harmless substance) or the alternative hypothesis (substance of concern) is usually performed by a statistical test, e.g., the Wilcoxon test (proof of hazard). Harmlessness is concluded if the p-value is greater than 5%, i.e. the null hypothesis of equal expected values is not rejected.

Decision Scheme

The type I error rate (α) is the false-positive rate, i.e., the probability of false rejection of H_0, while in "truth" no difference between treatment and control exists. The type II error rate (β) is the false-negative rate, i.e., the probability of erroneous retention (i.e., non-rejection) of H_0, although in "truth" a difference between treatment and control exists; see the following decision scheme (Table 1):

Proof of Hazard Approach

The common design in regulatory toxicology includes a negative control, several (commonly 2–4) dose groups, and sometimes a positive control. For normal distributed endpoints, such as organ weights, the US National Toxicology

Table 1 Error rates

		Computer output	Absolute, unknown truth	
			H_0 is true, i.e., no effect	H_0 is not true, i.e., effect
Test decision	H_0 not rejected	– (empty)	True	Type II error (β) false-negative rate
	H_0 rejected	*	Type I error (α) false-positive rate	True

This results in the two fundamental problems of the confirmatory test statistic: (i) only one of two errors – α or β – is directly controlled, and (ii) the second error can only be controlled indirectly by a priori sample size determination (statistically) or definition (regulatory). It follows that the hypothesis is formulated in such a way that the content's more meaningful error was chosen as type I error. Thus, there are two test options: (i) tests of efficacy, in the case in screening research with the direct control of false-positive rate (proof of hazard), and (ii) tests of equivalence (two-sided hypotheses) respective test of non-inferiority (one-sided hypotheses) with the direct control of false-negative rate (proof of safety). For toxicological studies, therefore two concepts exist (see Tables 2 and 3)

Table 2 Proof of hazard

Null hypothesis	$H_0^{\text{hazard}}: \mu_{\text{treatment}} - \mu_{\text{control}} \leq 0$ (substance harmless)
Alternative hypothesis	$H_A^{\text{hazard}}: \mu_{\text{treatment}} - \mu_{\text{control}} > 0$ (substance harmful)

Table 3 Proof of safety

Null hypothesis	$H_0^{\text{safety}}\ \mu_{\text{treatment}} - \mu_{\text{control}} \geq \delta$ (substance harmful)
Alternative hypothesis	$H_1^{\text{safety}}\ \mu_{\text{treatment}} - \mu_{\text{control}} < \delta$ (substance harmless)

Here $\delta > 0$ is minimal tolerable toxic effect, whereas we assume increasing values are toxic

Program recommends the use of either Dunnett (1955) or Williams (1972) procedure for pairwise comparisons between the dose group and the zero-dose control group. Both procedures control a familywise type I error (false-positive) rate. The first procedure tests chances against control, whereas the second tests a monotonic trend including control. As long as monotonicity can be assumed, the Williams test is the recommended test in the proof of hazard. When downturn effects at high doses are possible, the Dunnett test or a related Williams test modification robust against such specific non-monotonicity at high doses should be used. Through the multiplicity adjustment (control of familywise type I error rate), these tests however intensify the control of the actually less relevant false-positive rate substantially. For example, the false-negative rate increases from 17.6% to 34.4% when comparing three groups with a control by the Dunnett procedure compared with independent t-tests (endpoint body weight, to be detected difference $\Delta = 10$ g, $\sigma = 10$ g sample sizes 14, $\alpha = 5\%$, Bonferroni adjustment). Therefore, pairwise two-sample tests "control vs. dose," each at level α, may represent a compromise.

The evidence of a global trend represents a relevance criterion; on the other hand, the maximum safe dose (no observed effect level, NOEL) would be determined in toxicology also. The determination of a global trend appears as an easy task; nonlinear models or linear regression after data transformation is

used frequently. The dilemma is that these approaches much depend on the shape of the dose–response. But the shape is not an assumption; it is just an outcome of the experiment. Looking at data post hoc and selecting a particular model based on that impression is statistically incorrect. Therefore, it requires methods which are sensitive to all the possible shapes of the dose–response dependency. Tests with restriction order (trend test), based on the restricted alternative hypothesis,

$$H_0 : \mu_C = \mu_{D_1} = \mu_{D_2} = \ldots = \mu_{D_k}$$
$$H_A : \mu_C \leq \mu_{D_1} \leq \mu_{D_2} \leq \ldots \leq \mu_{D_k}$$

can be used where (at least one $\mu C < \mu Dk$) inequality in the alternative must hold true.

For this purpose, there are two principles: the likelihood ratio test and multiple contrast tests. Since the second approach is easier, numerically feasible confidence intervals are available, and power can be directly estimated; this should be shown here shortly. The Williams procedure is a special order-restricted test including the zero-dose control – an important argument for its use in toxicology. The idea will be illustrated with reference to the experimental design ([C, D_1, D_2]).

Here, precisely, there are two possible dose–response profiles with respect to the control:

$$H_A^1 : \mu_C = \mu_{D_1} < \mu_{D_2}$$
$$H_A^2 : \mu_C < \mu_{D_1} = \mu_{D_2}$$

For each profile, a contrast test can be used:

$$T_j = \frac{\sum_{i=C}^{k} c_i \overline{X}_i}{\sqrt{S^2 \sum_{i=C}^{k} \frac{c_i^2}{n_i}}}$$

The two sets of contrast coefficients c_i are (simplified for a design with equal sample sizes)

$$c_i^1 \; -1 \;\; 0 \;\; 1$$
$$c_i^2 \; -1 \;\; 0.5 \;\; 0.5$$

The maximum test

$$T_{max} = \max\left(T_1, \ldots, T_q\right)$$

Table 4 Ames assay data

I	Dose	Revertants	Mean
0	0	23, 22, 14	19.7
1	100	27, 23, 21	23.7
2	333	28, 37, 35	33.3
3	1000	41, 37, 43	40.3
4	3333	28, 21, 30	26.3
5	10,000	16, 19, 13	16.0

The following contrasts and their multiplicity adjusted p values result using the R code:
library(multcomp)
mymod<−lm(y∼Dose, data=salmonellaTA98)
summary(glht(mymod, linfct=mcp(Dose="Williams"), alternative ="greater"))
See Table 5

Table 5 Contrast coefficients and adjusted p values

Contrast	Coefficients' ci						p value
1	1	2	3	4	5	6	0.26
	−1	0	0	0	0	1	
2	1	2	3	4	5	6	0.095
	−1	0	0	0	0.5	0.5	
3	1	2	3	4	5	6	0.037
	−1	0	0	0.33	0.33	0.33	
4	1	2	3	4	5	6	0.069
	−1	0	0.25	0.25	0.25	0.25	
5	1	2	3	4	5	6	0.142
	−1	0.2	0.2	0.2	0.2	0.2	

For contrast 3, a slightly significant p value results, indicating a global significant trend, whereas a plateau including the doses 1000, 333, and 100 μg (contrast 3 with a p value of 0.037) is most likely (Fig. 1)

(in our example q = 2) is multivariate (q) distributed with a correlation matrix defined by the contrast coefficients and the sample size (using R library multcomp) or can be calculated by resampling approach (SAS procedure MULTTEST).

This approach is demonstrated by Ames assay data (Table 4).

However, the boxplots indicate an increase up to doses 100 μg; hereafter a downturn effect occurs. Therefore, the modified Williams procedure protected against downturn effect at higher doses is used (Bretz and Hothorn 2003). The idea is to test the global Williams trend together with a trend up to dose 333 μg, 100 μg, and 10 μg, i.e., for all possible peak points of the dose–response simultaneously (Table 6)

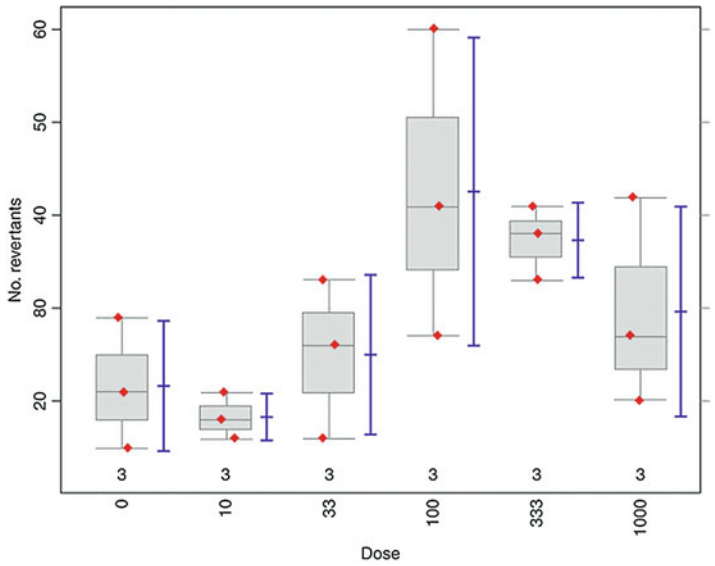

Fig. 1 Boxplots for Ames assay data

Proof of Safety Approach

The toxic response of most endpoints is either decreasing or increasing, such as declining numbers of offsprings in the Daphnia aquatic assay or rising number of micronuclei in the micronucleus assay. That is, the other direction is irrelevant for a toxicological perspective. Therefore, one-sided tests are appropriate for these assays. The harmlessness can be concluded by rejection of the null hypotheses that the difference between the treatment and dose effect is larger than an irrelevance threshold δ. This decision can be achieved by so-called non-inferiority tests. However, the a priori definition of irrelevance threshold δ is needed. Because this threshold is endpoint specific and scale dependent, a consensus is hard to find for different toxicological assays. For aquatic toxicity assays recently instead of difference to control, ratio-to-control tests were proposed (Denton et al. 2011) which allow a percentage, i.e., scale-independent definition of the threshold η. For chronic assays $\eta = 75\%$ and acute assays $\eta = 80\%$ were proposed. The approach provides several advantages: (i) the proof of safety concept controls the more important false-negative decision rate directly, (ii) it focuses on the toxicological relevant direction of decreasing effects, and (iii) it avoids the false claim of harmlessness when designs with insufficient small sample sizes are used (Hauschke et al. 1999).

The two-sample test for ratio-to-control comparison is a modification of the t-test (Fig. 2, Table 7):

Table 6 Contrast coefficients and adjusted p values

Contrast	Peak dose							
1	1000	1	2	3	4	5	6	0.42
		−1	0	0	0	0	1	
2		1	2	3	4	5	6	0.17
		−1	0	0	0	0.5	0.5	
3		1	2	3	4	5	6	0.071
		−1	0	0	0.33	0.33	0.33	
4		1	2	3	4	5	6	0.13
		−1	0	0.25	0.25	0.25	0.25	
5		1	2	3	4	5	6	0.25
		−1	0.2	0.2	0.2	0.2	0.2	
6	333	1	2	3	4	5	6	0.12
		−1	0	0	0	1	**0**	
7		1	2	3	4	5	6	**0.038**
		−1	0	0	0.5	0.5	**0**	
8		1	2	3	4	5	6	0.104
		−1	0	0.33	0.33	0.33	**0**	
9		1	2	3	4	5	6	0.25
		−1	0.25	0.25	0.25	0.25	**0**	
10	100	1	2	3	4	5	6	0.04
		−1	0	0	1	**0**	**0**	
11		1	2	3	4	5	6	0.16
		−1	0	0.5	0.5	**0**	**0**	
12		1	2	3	4	5	6	0.39
		−1	0.3333	0.3333	0.3333	0	0	
13	33	1	2	3	4	5	6	0.68
		−1	0	1	**0**	**0**	**0**	
14		1	2	3	4	5	6	0.88
		−1	0.5	0.5	**0**	**0**	**0**	
15	10	1	2	3	4	5	6	0.93
		−1	1	**0**	**0**	**0**	**0**	

Contrast 7 reveals the smallest p value, i.e., the peak dose is 333 μg (not the 100 μg guessed from the boxplot), and a plateau including doses 333 and 100 μg is most likely

A different objective is the estimation of the NOEL. Commonly it is estimated by step-down significant trend tests, whereas NOEL is the next lower dose after the last significant trend test. But this classic proof of hazard has the disadvantage that with small sample sizes, lower doses would be characterized as safe. An alternative concept of maximal safe dose (MAXSD) is described in the following section for the proof of safety

$$T^{\text{Ratio}} = \frac{\overline{X}_T - \overline{X}_C}{\sqrt{S^2\left(\frac{1}{n_T} + \frac{2}{n_C}\right)}}$$

whereas the p values for the two-sample tests for the concentrations 1.56, 3.12, and 6.25 are very small, i.e., these concentrations are harmless; the p value for the concentration 6.25 is $p = 0.99$, i.e., this concentration is not harmless; see the R code:

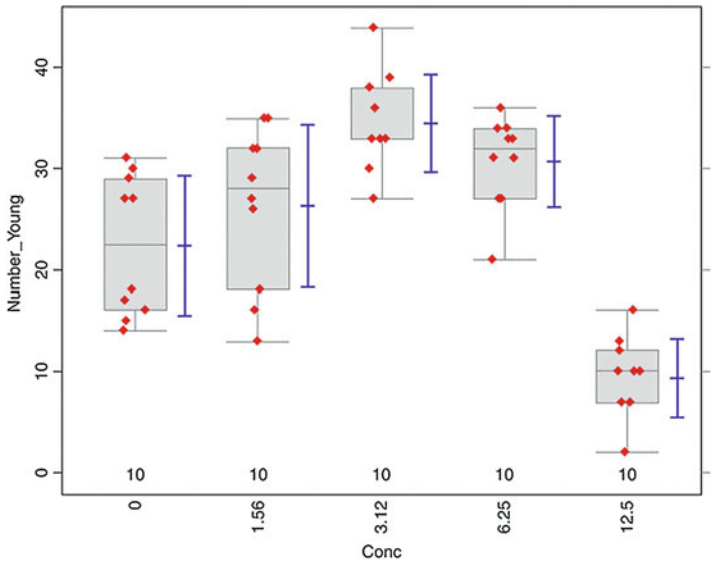

Fig. 2 Boxplots for Daphnia data

Table 7 Reproduction data for the daphnid *Ceriodaphnia dubia* exposed to an effluent for 7 days

Concentration/%	No. young per adult
Control	27, 30, 29, 31, 16, 15, 18, 17, 14, 27
1.56	32, 35, 32, 26, 18, 29, 27, 16, 35, 13
3.12	39, 30, 33, 33, 36, 33, 33, 27, 38, 44
6.25	27, 34, 36, 34, 31, 27, 33, 21, 33, 31
12.5	10, 13, 7, 7, 7, 10, 10, 16, 12, 2
25.0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0

```
library(mratios)
t.test.ratio(Number_Young~Conc, data=daph, rho=0.8, base=1,
alternative="greater," var.equal=TRUE)
```

The estimation of the maximally safe dose (MAXSD) can be performed by step-up non-inferiority tests (Table 8)

Impact of Sample Size

The sample size has a central position in decisions of tests, because the secondary error rate is determined only by an a priori estimation of the sample sizes. This will be illustrated for the proof of hazard and proof of safety on the basis of parametric tests (see Table 9).

Table 8 Decision tree Daphnia example

Step	Comparison	p value	Decision
1	$\mu_{1.56}/\mu_{control}$	6.9e−04	Significant, i.e., harmless
			Go to step 2
2	$\mu_{3.12}/\mu_{control}$	2.5e−10	Significant, i.e., harmless
			Go to step 3
3	$\mu_{6.25}/\mu_{control}$	4.9e−07	Significant, i.e., harmless
			Go to step 4
4	$\mu_{12.5}/\mu_{control}$	0.99	Not significant, i.e., not harmless, i.e., dose 6.25 is MAXSD
–	$\mu_{25.0}/\mu_{control}$	Not tested	–

The related R code is the following:

```
library(mratios)
simtest.ratio(Number_Young~Conc, data=daphnia, type="Dunnett," alternative="greater,"
Margin.vec=c(0.8,0.8,0.8,0.8))
```

Table 9 Estimated sample sizes

Proof of hazard		Proof of safety		
Detectable difference Δ	Sample size	$CV_{Control}$	k-fold threshold η	Sample size
2.5 g	199	10%	0.8	8
5 g	51		0.667	3
7.5 g	23		0.5	(1)
10 g	14	25%	0.8	28
12.5 g	9		0.667	10
15 g	7		0.5	5
17.5 g	5	50%	0.8	109
20 g	4		0.667	35
25 g	(1)		0.5	14

For example, for the terminal body weight, a standard deviation estimator from historical studies of $\sigma = 10$ g and normal distribution can be derived

For the proof of hazard approach, the necessary sample size per group can be estimated for several detectable differences Δ (one-sided *t*-test, design with equal sample sizes, type II error rate = 20%, type I error 5%); see Table 9

For the proof of safety using the ratio-to-control test, the sample sizes depend on the coefficient of variation of the control ($CV_{Control}$) and the irrelevance threshold η. Sample size depends seriously on the accuracy requirements. Therefore, sample size should be chosen "per chance" but by either a statistical calculation or regulatory recommendation

Presentation of Significances

The results of significance tests, such as the Dunnett test, are often represented by asterisks (* . . . $p < 0.05$, ** . . . $p < 0.01$) or directly by p values in tables or graphs, since it is highly condensed and clearly structured. With the common-used formal p value threshold of 0.05, a dichotomous decision is derived into "significant" and

"non-significant." The p value is a probability that cannot illustrate the different influences of effect size (e.g., mean difference) and uncertainty (a function of variance, sample size, false-positive rate, and other factors). In risk assessment, "absence of evidence is no evidence of absence" (Altman and Bland 1995) is a further issue hereby. An alternative is confidence intervals, best used as compatibility intervals (Hothorn and Pirow 2020). No formal 95% level is used, and their compatibility with the data and model assumptions is considered. As an example, the analysis of the pup weights of a reproductive toxicology study is considered. On the one hand, the per-litter structure is represented by a mixed effect model, in which possible gender differences and litter sizes are also modelled. From this, adjusted p values of a Dunnett-type test (Hothorn 2015) can be integrated into a boxplot (Fig. 3) (Pallmann and Hothorn 2015), or 90% two-sided intervals can be used (Fig. 4), indicating that even at the low dose is a weight retardation, which becomes relevant at the high dose.

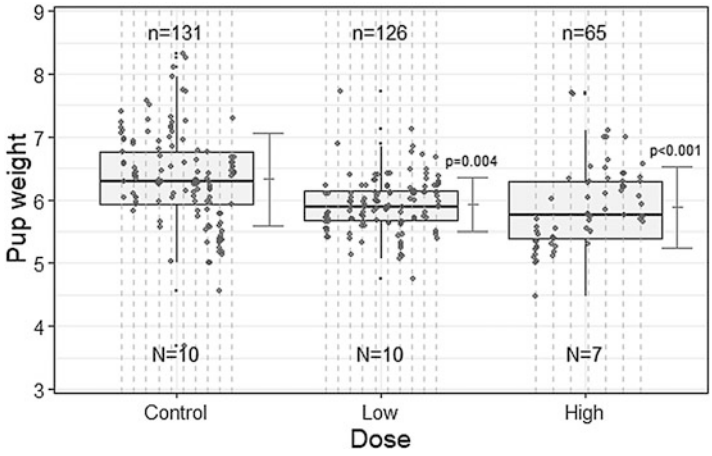

Fig. 3 Boxplots for pub weight data visualizing the litter structure and the p values

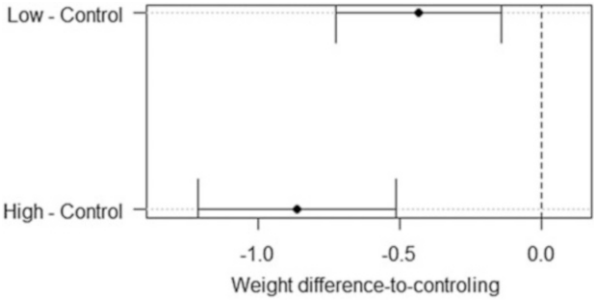

Fig. 4 Simultaneous, two-sided intervals for difference to control pup weights, adjusted for sex, litter structure, and litter size

The Dunnett Test and Its Modifications

The Dunnett test is the standard test in regulatory toxicology. Initially, it is defined for normally distributed, variance homogeneous errors and offers both adjusted p values and confidence intervals compatible. It controls the familywise error rate and thus has an inherently higher false-negative rate than pairwise level alpha tests. Variance heterogeneity is rather the typical result of an assay, and therefore the use of modifications with a robust variance estimator (Herberich et al. 2010) or a reduced degree of freedom (Hasler and Hothorn 2008) is strongly recommended. Unfortunately, these show an increased false-negative rate, especially in unbalanced designs. Therefore, designs with approximately equal n_i are recommended. Sometimes a global analysis of variance and/or pretests for normal distribution, variance homogeneity, or outliers are performed before the Dunnett test. This is problematic, especially with the usual small n_i. Either one relies on the robustness of the test, or one uses the nonparametric variant (Konietschke and Hothorn 2012) or a robust modification based on most likely transformation models (Hothorn and Kluxen 2019). Different scales are typically in toxicology (Szoecs and Schaefer 2015). Therefore, appropriate modifications of the Dunnett test should be used, for proportions (such as rates) (Schaarschmidt et al. 2008), for counts (Hothorn et al. 2013; Jaki et al. 2014)), for mortality adjusted poly-k estimators (tumor rates) (Schaarschmidt et al. 2008a), for survival functions (Herberich and Hothorn 2012), and for correlated multiple endpoints (Hasler and Hothorn 2011). The open-source project for statistical computing R, the packages multcomp, mratios, MCPAN, and SimComp simplify the calculation considerably.

Cross-References

- ▶ Benchmark Dose Approach in Regulatory Toxicology
- ▶ Dose–Response Relationship and Extrapolation in Toxicology: Mechanistic and Statistical Considerations
- ▶ Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds
- ▶ Uncertainty Analysis in Exposure Assessment-Relevance for Toxicological Risk Assessment

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Dose-Response Analysis: Identification of Threshold Levels for Chemicals

22

Hans-Karl Heim and Peter Mayer

Contents

Abstract

Following the initial observation of a toxic effect of a substance in humans or animals ("hazard identification"), the determination of the dose-effect relationship for the observed toxic effect represents the next step. The toxicological

H.-K. Heim (✉) · P. Mayer
Federal Institute for Drugs and Medical Devices, Bonn, Germany
e-mail: Hans-Karl.Heim@bfarm.de; Peter.Mayer@bfarm.de

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threshold levels identified in this process (e.g., no-observed-adverse-effect level, NOAEL) are then used for a quantitative toxicological risk assessment, taking into account the available exposure data.

Keywords

Allometric scaling · Dissociation constant · Dose-response relationship · Efficacy · Exposure multiple · Hormetic curve · Intrinsic activity · Law of mass action · Lethal dose 50 (LD_{50}) · Liver hypertrophy · Lowest-observed-adverse-effect level (LOAEL) · Lowest-observed-effect level (LOEL) · No-observed-adverse-effect level (NOAEL) · No-observed-effect level (NOEL) · Potency · Receptor agonist · Receptor antagonist · Risk assessment · Therapeutic ratio · Thyroid hypertrophy · Toxicological threshold

Introduction

Following the initial observation of a toxic effect of a substance in humans or animals "hazard identification," step 1 of the National Academy of Sciences (NAS)-scheme (National Research Council, US 1983), the determination of the dose-effect relationship for the observed toxic effect represents the second step in the NAS-scheme. The toxicological threshold levels identified in this process (e.g., no-observed-adverse-effect level) are then used for a quantitative toxicological risk assessment (NAS step 4) on an individual and/or collective level, taking into account the available exposure data (NAS step 3).

Toxic Effects on the Individual Level

Interaction of Toxicants with Target Molecules in Living Organisms

Pharmaco-toxicological effects of chemical compounds, whether desired (e.g., in the case of therapeutic effects of medicinal products; see also Blumenthal 2011) or unwanted (e.g., in the case of detrimental effects of environmental toxicants), result in most cases from an interaction with specific target molecules in living organisms.

An important exemption from this rule are chemically reactive compounds or compounds from which reactive metabolites (e.g., free radicals) are formed, which modify biomolecules more or less unspecifically. In this case, the resulting toxicological effects are dependent on numerous factors, for example, the regenerative or repair capacity of the affected cell or organism, so that no simple model exists to describe the pharmaco-toxicological effects of such compounds.

However, usually defined macromolecules, for example, proteins or nucleic acids, are the specific targets of toxicants. The interaction of a toxicant/drug with its target(s) is in most cases mediated by binding to the target molecule, which is characterized by a specific affinity. In the case of toxic compounds, the normal

physiological function of the target molecule is usually affected by this process, whereby toxic (pathophysiological) consequences can be induced.

Reversible and Irreversible Damage, Accumulation of Toxic Effects

A toxicant may bind to its target either in a reversible (e.g., by ionic or van der Waals binding) or irreversible (e.g., by covalent binding) way. However, whether a toxic effect is in the end reversible or irreversible does not only depend on the kind of interaction of the toxicant with its target molecule but also on the capacity of the respective target tissue for regeneration.

For example, the covalent, irreversible inactivation of the enzyme cholinesterase by a sublethal dose of the insecticide parathion (E605) may not necessarily lead to an irreversible damage, since new cholinesterase is continuously synthesized by the organism, whereby the toxic effect of parathion may be reversed. Also in the case of toxic effects on the liver, an organ with a high capacity for regeneration, tissue damage induced by toxicants is often reversible. On the other hand, a toxicant-induced damage of the peripheral or central nervous system is often irreversible, since differentiated nervous cells have no or only limited capacity for regeneration (e.g., irreversible damage of the sensory cells of the inner ear by aminoglycoside antibiotics with permanent deafness/hardness of hearing as consequence).

In the event of irreversible effects, not only the administered single dose is important but also the cumulative dose which is taken up during lifetime (summation toxicants). For example, for the anthracycline derivative doxorubicin (used as a cytostatic), the cumulative total lifetime dose should not exceed 450 mg/m^2 body surface, since for cumulative doses exceeding this empirically defined value, the risk of manifest heart damage is strongly increasing. In practice, threshold levels can be identified for many irreversible toxic effects.

Receptor-Mediated Toxic Effects, Law of Mass Action, and K_D-Value

Hormone and neurotransmitter receptors represent important targets for many toxicants. In case of receptor-mediated toxic effects, the intensity of these effects depends on the number of receptors that are affected by the toxicant. The receptor affinity of the toxicant (ligand) determines its potency. The higher the affinity, the higher the number of occupied receptors at a given concentration of the ligand. The affinity is quantitatively described by the so-called dissociation constant K_D. This constant describes the disintegration of the receptor-ligand complex (RL) in case of reversible interactions:

$$RL \rightarrow R + L \tag{1}$$

This process is the reversion of the binding reaction of the ligand to its receptor (which is often easier to determine experimentally than the forward reaction of binding). Since the reaction obeys the law of mass action, the dissociation constant is given as

$$K_D = \frac{[R]\ [L]}{[RL]} \tag{2}$$

where [L], [R], and [RL] are the concentration of the free ligand, the receptor, and the ligand-receptor complex, respectively. The K_D -value indicates at which ligand concentration half of the receptors are occupied. The *smaller* the K_D, the *higher* the affinity of the ligand to its receptor.

Sigmoid Shape of Toxicant-Receptor Binding Curves

The magnitude of the pharmaco-toxicological effect of a ligand depends on the amount of ligand-receptor complex formed, that is, [RL], because usually only a receptor that carries a bound ligand is biologically active. [RL] depends on the ligand concentration [L] in a way that can be derived by transformation from Eq. 2:

$$[RL] = \frac{[R]_t[L]}{K_D + [L]} \tag{3}$$

In this equation, $[R]_t$ stands for the total receptor concentration, that is, for the sum of free receptor and receptor carrying ligand, [R] + [RL]. When [RL] is plotted against [L] according to the function provided in Eq. 3, then a hyperbolic curve results (Fig. 1a).

With a logarithmic scale of the x-axis, a sigmoid shape of the curve results (Fig. 1b). This logarithmic presentation more clearly indicates that a significant amount of ligand-receptor complex is only formed when the ligand concentration exceeds a certain limit (in the example of Fig. 1b a ligand concentration above around 10^{-7} mol/L). For smaller ligand concentrations, formation of ligand-receptor complexes and therefore biological activity is virtually negligible. The K_D-value can be estimated by determining the concentration that elicits the half-maximal response. This concentration is called EC_{50} and is equal to K_D as long as the assumptions made above are valid, that is, that the effect size is only determined by [RL] and that [RL] only depends on the affinity of the ligand to its receptor. Derivations from this ideal case can occur, for example, if the receptor has additional binding sites for other ligands, leading to allosteric effects, or if there are more receptors than signaling molecules (so-called spare receptors) so that not every occupied receptor can contribute to the effect.

Toxicant-Receptor Binding and Agonistic and Antagonistic Effects, Intrinsic Activity

A ligand, which is bound to its receptor, may activate (receptor agonist) or block (receptor antagonist) this receptor. Both processes may result in either desired or

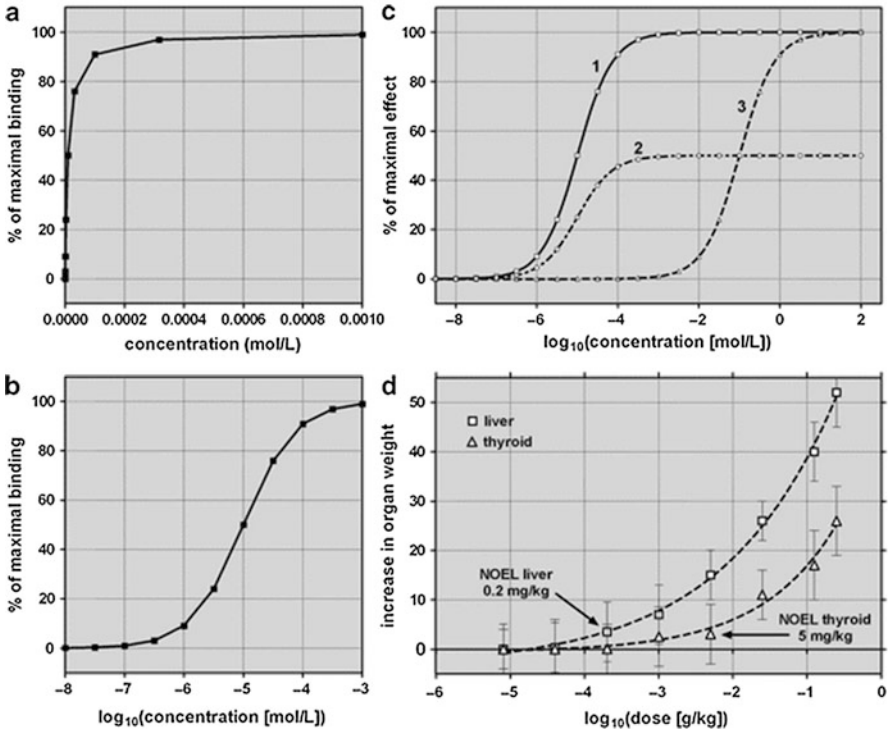

Fig. 1 Ligand binding, dose-response relationship, and toxicological threshold levels. (**a**) shows the relationship between the ligand concentration and the resulting extent of binding of the ligand (e.g., a toxin) to the receptor. The relationship follows the law of mass action. The graphical view on a linear x-axis scale yields a hyperbolic curve. (**b**) shows the same as (**a**) but now with a logarithmic display of the ligand concentration. A sigmoid curve results. (**c**) shows dose-response relationships for ligands with different potency (substance 1 = substance 2 > substance 3) and different efficacy (substance 1 = substance 3 > substance 2). In (**d**), the deduction of the NOEL level for the effects of a substance on the organ weights of liver and thyroid is visualized. Shown are mean values ± 95% confidence interval of replicated experiments. At the NOEL, the effect size is not significantly different from zero. See text for further explanations

unwanted effects for the organism. A classic example for a toxic agonistic effect is observed for the mushroom poison muscarine, which can result in an over-stimulation of the parasympathetic nervous system by an activation of cholinergic receptors. Contrarily, atropine, an alkaloid contained in belladonna, can elicit severe toxic effects by an antagonistic interaction with cholinergic receptors. However, atropine, by virtue of its cholinergic receptor-blocking properties, can be used as an antidote in case of intoxication with muscarine.

While muscarine is designated as an *agonist* at certain cholinergic receptors, atropine is designated as an antagonist. More specifically, atropine is called a direct antagonist of muscarine, because it binds to the same receptors. On the other hand, atropine may indirectly abrogate, as a functional antagonist, the decrease in heart rate

induced by ß-receptor antagonists, by blocking inhibitory effects of the parasympathetic nervous system on heart rate (for completion of definitions: a chemical antagonist can abrogate the effects of another substance by direct chemical inactivation, for example, dimercaptopropane sulfonic acid in the case of toxic effects of lead).

A receptor ligand may act as an agonist or as an antagonist. This behaviour is described by the so-called intrinsic activity, which quantifies the potential to activate, via binding to its receptor, downstream signal transduction mechanisms in the target cell (e.g., the cAMP system). It is possible to differentiate between "full agonists," which result in a maximal activation (intrinsic activity = 1) and *partial agonists*, which only result in a submaximal activation of signal transduction mechanisms (intrinsic activity >0 but <1). Pure antagonists have an intrinsic activity of 0; the so-called inverse agonists have an intrinsic activity <0.

Characterization of Toxicological Efficacy and Potency

The considerations outlined above assumed that the complete amount of administered ligand is directly available for receptor interaction, as it is the case in vitro. In vivo there is also usually a positive correlation between the total dose administered to the organism, the plasma concentration, and the concentration in the compartment where the receptor is located (often in a tissue outside the vasculature, either on the cell surface or intracellularly). Ideally, there is a linear relationship between the administered dose and the resulting ligand concentration [L] in the vicinity of the receptor. Therefore, the relationship between ligand concentration and response given in Eq. 3 can in principle also be applied to characterize the relation between administered dose and response.

The term efficacy describes the maximal effect (E_{max}) a substance can elicit if administered in sufficient dose. This situation is usually reached if all available receptor molecules have bound a ligand molecule, that is, in the case of maximal binding (B_{max}). A further increase of dose (and therefore of [L]) is not capable to increase the effect size further. In the graphical presentation of the dose-response relationship, this is reflected by the leveling off of the sigmoid curve at high ligand doses. Depending on the intrinsic activity of the ligand at its receptor, its efficacy may differ. By definition, full intrinsic activity is achieved with an agonist which activates the receptor to the highest extent possible. In the example of Fig. 1c, substance 2 shows a lower efficacy (intrinsic activity = 0.5, designated as partial agonism), whereas substances 1 and 3 display full agonistic activity (intrinsic activity = 1).

The potency of a ligand expresses how much (respectively how little) of this substance is needed to elicit a certain magnitude of response (usually the half-maximal response). The lower the required dose, the higher the potency. A higher potency manifests itself graphically as a shift to the left of the dose-response curve. In the example of Fig. 1c, substances 1 and 2 possess a higher potency than

substance 3 because the half-maximal effect (ED_{50}) is already reached at a dose of 10^{-5} mol/L, whereas for substance 3 the ED_{50} is 10^{-1} mol/L.

Identification of NOEL and LOEL as Toxicological Threshold Levels

Toxicants can have pharmaco-toxicological effects on different organs, for each of which a separate dose-response relationship can be established. This is illustrated in Fig. 1d, which shows the increase in organ weight of liver and thyroid in relation to the administered dose of a test substance. An increase in liver weight, often accompanied by histopathological signs of hyperplasia, is a relatively frequent toxicological finding.

This is because foreign substances often induce the expression of drug metabolizing enzymes in the liver, an effect that in turn may be associated with liver cell hypertrophy and hyperplasia in the long term. Thyroid hypertrophy on the other hand may be caused either by a direct thyrostatic effect of a test substance or may occur secondary to enzyme induction in the liver, because the induced enzymes may degrade thyroid hormones more intensively and the thyroid gland has to produce a higher amount of hormones to keep the thyroid hormone plasma levels constant. Nevertheless, even under these circumstances, liver and thyroid hypertrophy can display different dose-response relationships. This is because a considerable extent of enzyme induction in the liver is often necessary until first signs of secondary thyroid hypertrophy may become evident, and there may be toxicant/drug doses at which an increased liver weight is found but no effects on thyroid weight can yet be observed.

The lowest dose at which an effect is observed is called the LOEL (lowest-observed-effect level). In the example shown in Fig. 1d, a statistically significant effect (organ weight increase) was observed at a dose of 1 mg/kg or above in the liver but not until 25 mg/kg in the thyroid.

The NOEL (no-observed-effect level) is the tested dose level just below the LOEL. In the example provided in Fig. 1d, the NOEL was 0.2 mg/kg for liver and 5 mg/kg for thyroid. It should be emphasized that the LOEL (and therefore the NOEL) is usually defined by a statistically significant effect at this dose level although small, not statistically significant effects may be obvious already at lower dose levels. Furthermore, although in Fig. 1d a curve is fitted on the data points for clarity, fitted curves are usually disregarded for LOEL/NOEL determination and only the actual data points considered relevant. It should be pointed out that for statistical calculations, the number of replicates (animals per group in this case) and the statistical model used are important parameters. In the example shown in Fig. 1d, each dose level is regarded separately and statistical significance assumed if the 95% confidence interval (95% CI) no longer contains zero. Other approaches would also be conceivable, for example, a trend analysis. In any case, the selection of the statistical model used has to be justified.

Problems in Determination of NOEL and LOEL Values

On basis of the aforementioned considerations, it is obvious that the calculated NOEL and LOEL values, respectively, will reflect the true "no effect level" and "lowest effect level" all the better if the number of dose levels evaluated and the number of measured values per tested dose level (i.e., in animal studies, the number of evaluated animals) is high, because then statistical significance can already be reached for small deviations from the control value. In case that only few measured values are available, statistical uncertainty may be so high, that a NOEL can only be assigned far in the ascending part of the curve. To avoid this, toxicity studies should be performed with a sufficient number of animals. For example, for performance of nonclinical chronic toxicity and carcinogenicity studies in rodents in context with marketing authorization of medicinal products, the use of at least 20, respectively, 50 animals per gender and dose level is recommended in OECD Guidelines 451 and 452 (see OECD 2020).

Reporting of NOAEL and LOAEL Values

Particularly during assessment of nonclinical study data for medicinal products, it may, in certain circumstances, be a matter of discretion: which effects should be considered as unwanted (and therefore be avoided) and which effects can be tolerated with regard to the therapeutic benefit of the medicinal product. In the example shown in Fig. 1d, it would be conceivable to tolerate liver hyperplasia, if this is not related to irreversible liver damage, however, to consider impairment of thyroid function as being not tolerable.

For effects which are regarded as disadvantageous for the individual, instead of the NOEL value (which includes desirable effects), the NOAEL (no-observed-adverse-effect level) is given. Accordingly, the lowest dose level at which a significant adverse effect is observed is designated as LOAEL (lowest-observed-adverse-effect level). In the example presented in Fig. 1d, the NOAEL would be 5 mg/kg, if liver hyperplasia is considered as "not adverse," and 0.2 mg/kg, if it is considered as "adverse."

Toxic Effects in Collectives

Differences in the Individual Sensitivity

In the preceding paragraphs, the dose dependency of toxicant/drug effects was described, which should in principle be valid for each individual of the evaluated species. However, it should be considered that not each animal or human being will react in an exactly identical way to a given toxicant/drug. In practice, some individuals will react more sensitive and others will react less sensitive than the average. For

the more sensitive individuals, a given toxic effect will therefore occur already at a lower toxicant dose when compared with the less sensitive individuals.

LD_{50} and TD_{50}

The dose-response relationship in collectives shall be exemplified for a long-known toxicological parameter, the LD_{50} (LD stands for lethal dose), which indicates the dose of a test substance at which half of the treated animals die. The LD_{50} is a first, orientating but rather crude measure for the toxicity of a substance. Ideally, differences in the individual sensitivity of the animals toward the substance result in a bell-shaped Gaussian curve for the lethal dose (see Fig. 2a). Few animals die already at a rather low dose. With increasing doses, the mortality rate reaches a maximum incidence (at the LD_{50}), and again few animals survive until a rather high dose is given. Integration of the Gaussian distribution results in a function *of* cumulative mortality versus dose which expresses the fraction of animals that die *until* (instead of *at*) a given toxicant/drug dose. This integrated Gauss curve again has a sigmoid shape, and, in analogy to the determination of ED_{50} from the dose-response curve, LD_{50} can be determined (graphically or with the help of an adequate computer program) from this function (see Fig. 2b). In toxicological tests, usually different groups of animals are treated with different, ascending doses of the test substance, and the event rate (in case of LD_{50} the event "death") is counted in each group. This approach immediately yields the integrated Gauss function. Similar calculations can be performed for other parameters of interest, for example, organ toxicity, behavioral changes, etc. In this case, the resulting quantitative parameter is not called LD_{50} but TD_{50} (TD stands for *toxic dose*).

Distribution of Individual Sensitivity, Therapeutic Range

The TD_{50} is an orientating parameter which gives no information about the interindividual variability of the effect. In case of a large variability, unwanted effects can occur in a relatively large part of the population already at doses that are far below the TD_{50}. This is visualized in Fig. 2c; there the distribution of the desired (therapeutic) effect and the unwanted (toxic) effect of a drug is plotted. For the toxic effect, a TD_{50} of 10^{-3} g/kg is identified, whereas the ED_{50} lies at 10^{-7} g/kg, which implies a large safety margin, with a therapeutic ratio (defined as quotient TD_{50}/ED_{50}) of 10^4. However, a more detailed consideration of the dose-response relationships reveals that there is considerable overlap of the therapeutic and toxic effect curves and that a considerable fraction of the total population experiences toxic effects already at doses needed to elicit the full desired effect. Quantitatively spoken, the dose that elicits the desired effect in 95% of the population (ED_{95}) also leads to toxic effects in more than 5% of the collective (shaded area in Fig. 2c), that is, is higher than the TD_5 value (Fig. 2d).

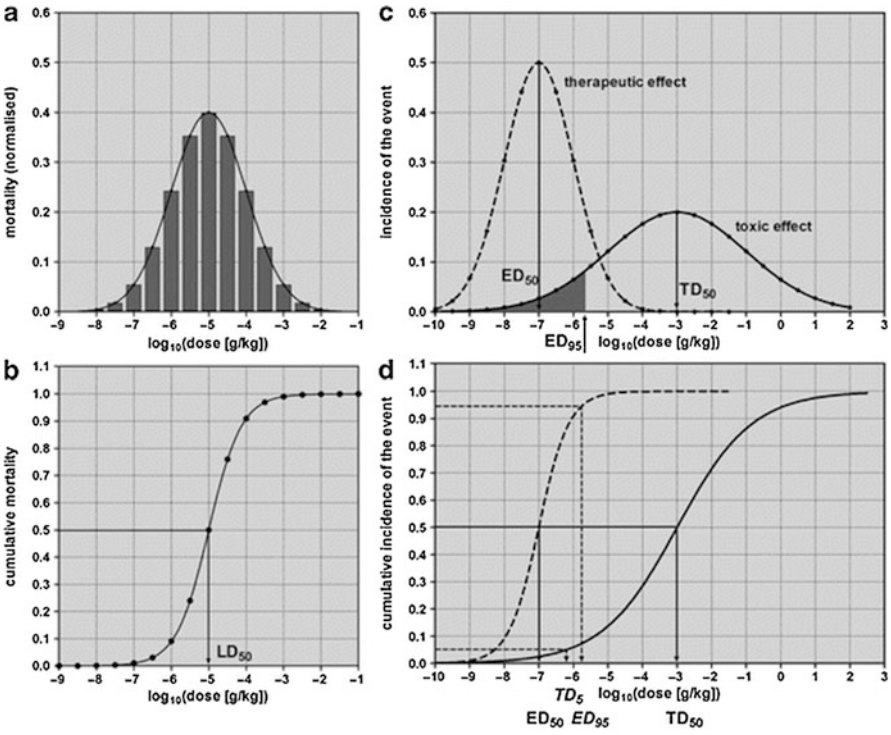

Fig. 2 Dose-response relationship in groups (collectives). (**a**) displays the statistical fluctuation of the individual lethal dose of a toxin within a collective. Ideally, as shown, a typical Gaussian distribution results. The maximum of the Gauss curve lies at the mean lethal dose (LD_{50}); in (**b**) the curve of cumulative mortality is shown which was obtained by integrating the curve displayed in part (**a**). From this cumulative presentation, the LD_{50}-value can be derived. (**c**) shows the frequency distribution, in relation to the dose, for a desired therapeutic effect and for an unwanted toxic effect. From the large interindividual variability, an overlap results, so that in some individuals (*shaded area*) toxic effects already occur at doses in the therapeutic range (i.e., up to ED_{95}). (**d**) provides the data shown in (**c**) in a cumulative, that is, integrated, manner with deduction of the parameters TD_5 and ED_{95} together with the standard parameters TD_{50} and ED_{50}.

Thus, a more safety-related definition of the therapeutic ratio uses the TD_5/ED_{95} quotient instead of the TD_{50}/ED_{50} quotient. In the example shown in Fig. 2c, d the TD_5/ED_{95} ratio is about 0.25 and thus markedly below 1.

Specific Dose-Effect Relationships

Dose-response curves with a U-shape (hormetic curves) can occur, for example, in the case of essential nutrients or trace elements. For example, vitamin A, when given in high doses, has a marked teratogenic effect. However, since vitamin A in a low dosage is essential for the correct embryo-fetal development, vitamin A deficiency may also result in the occurrence of malformations.

Extrapolation of Threshold Levels to Application in Humans

NOAEL and other threshold levels determined in animal studies are valid at first only for animals of the investigated species. Extrapolation of these threshold levels to the human situation in context of a marketing authorization procedure for a medicinal product is shortly exemplified below.

Use of More Than One Animal Species

According to the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals, ICH (ICH 2020), and European guidelines for evaluation of repeated dose toxicity of medicinal products, toxicological tests should usually be performed in more than one animal species, preferably both in a rodent and a non-rodent species. Hereby it is ensured that potential species-specific effects, that is, toxic effects that only occur in a specific animal species, are revealed. Often, such effects are not representative for human toxicology.

Quantification of Exposure

Usually, dosage in toxicological studies makes reference to the bodyweight (mg/kg bodyweight) of the used laboratory animals. However, a direct extrapolation of these values to the human situation may be problematic, since in smaller animals, at the same weight-based dosage, often a considerably lower systemic exposure than in larger animals or in humans is achieved (see Fig. 3). In such cases, standardization of the dosage to body surface (mg/m^2) often provides a better correlation (allometric scaling). However, instead of relying on data extrapolation to estimate exposure, it may be more favorable to measure the actual systemic exposure of experimental animals and of humans directly, for example, by determination of plasma levels following drug administration (see below).

Toxic Threshold Levels and Safety Margin in Humans

For marketing authorization of medicinal products, it is recommended to collect pharmacokinetic data in humans and in laboratory animals for comparison. Under the assumption that at a systemic exposure at which toxic effects are observed in the animal study (LOAEL), toxic effects can also be expected to occur in humans, a safety margin can be estimated for drug application to humans. For this purpose, the ratio of the systemic exposure (*area under curve, AUC*) at the LOAEL (or alternatively the NOAEL) in the animal study and the systemic exposure at the (maximal) therapeutic dose level in humans is calculated. This quotient is called exposure multiple and indicates how far the dose range, to which humans are exposed during therapeutic dosing of a medicinal product and the dose range, at and above which

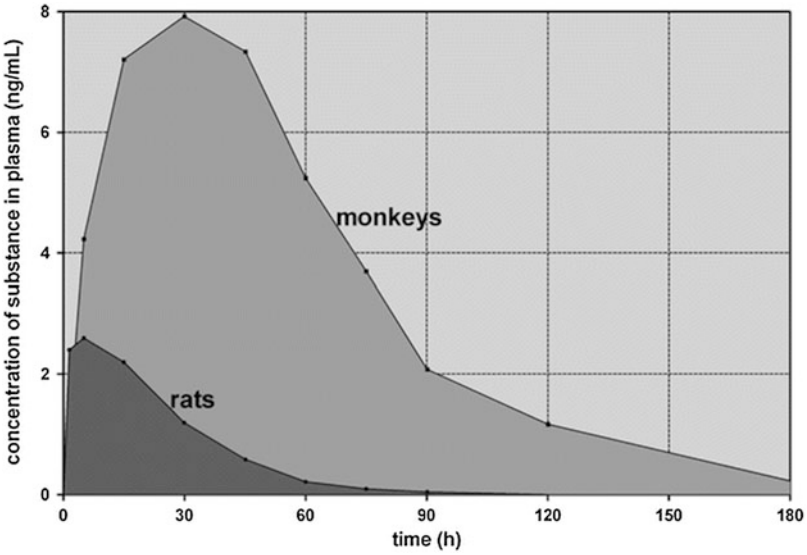

Fig. 3 Measurement of plasma levels for determination of drug exposure. The plasma levels (means) observed at different time points after single subcutaneous administration of a substance at a dose of 10 mg/kg at $t = 0$ are shown for rats and monkeys. Note that the maximal plasma levels (C_{max}) and the area under curve (AUC), reflecting integration of plasma levels over time and hence total systemic exposure, are much larger in monkeys (AUC 26 ng h/mL) than in rats (AUC 1.4 ng h/mL) in this example

toxic effects have to be feared, are separated. In practice, often additional factors have to be taken into consideration, for example, the fact that pharmacokinetics may show dose dependency, may change after repeated administration (e.g., by induction of enzymes involved in drug degradation/metabolism), or may be influenced by the gender or by genetic polymorphisms.

By taking into account the aforementioned parameters, finally, a risk-benefit analysis is performed for the medicinal product in which the main toxicological findings, the calculated safety margin, the expected therapeutic benefit, and specific factors related to the exposed patient population, are taken into consideration. A more detailed description of the benefit-risk evaluation is provided in other chapters of this book.

Cross-References

- ▶ Biomolecules Versus Smaller Chemicals in Toxicology: ICH, EU, and US Recommendations
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► Intrinsic Toxicity of Substances: Aspects for Risk Assessment
► Toxicological Risk Assessment
► Uncertainty Analysis in Exposure Assessment-Relevance for Toxicological Risk Assessment
► Working Areas of Regulatory Toxicology

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Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds

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Lutz Edler

Contents

Abstract

One fundamental goal of regulatory toxicology is to establish safe levels of human exposure to toxic compounds. This is usually performed within the framework of risk assessment and risk management. Using both data of human exposure (*exposure assessment*) and from the characterization of the toxicity (*hazard characterization*), the risk of the investigated compound or agent can then be characterized (*risk characterization*) in a framed approach through health-based guidance values (HBGVs) or related measures. In the absence of information to establish dose–response relationships at exposure levels such low as they are generally experienced by humans, high-dose to low-dose extrapolation has been used. Whereas epidemiological findings of the agent's toxicity are

L. Edler (✉)
Dudenhofen, Germany

Formerly: Division of Biostatistics, German Cancer Research Center-DKFZ, Heidelberg, Germany
e-mail: lutzedler@gmx.de

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directly applicable to humans, toxicological results detected in experimental animals need in addition the extrapolation from the specific animal species to human (interspecies extrapolation). To estimate the magnitude of expected effects at lower doses associated with real human exposure or corresponding to acceptable risks for humans set by regulatory practices, appropriate extrapolation is required. This chapter focuses on the low-dose extrapolation of animal data but presents at the same time general methodology also applicable to human data to establish HBGVs for humans.

Keywords

Risk extrapolation · Limit risk · Threshold · Point-of departure · Benchmark dose

Introduction

The fundament of the regulation of carcinogenic and noncarcinogenic compounds for human health protection is the toxicological and epidemiological knowledge base available when a specific agent or compound, often a chemical, is considered for risk assessment. Whereas epidemiological findings are directly applicable to humans they often are based on knowledge that was not obtained for the relevant dose range for the assessment. Toxicological data from the huge reservoir of experimental animals allow a designed approach to fill this gap, in principle. However, their results cannot directly be translated to humans because of systemic biological differences between the species. Well studied are differences in toxicokinetic and toxicodynamics. Furthermore, practical restrictions of the experiments (number of animals tested, duration of experiments, etc.) and the need to extrapolate between animals and humans may leave similar gaps regarding dose ranges as with epidemiological studies. Various extrapolation methods have been developed and applied to close the gaps and to estimate occurrence and size of adverse health effects expected at the low dose range relevant for humans. An outstanding organizing principle in the development of methods for extrapolation to low doses and decision making on exposure limits has been the distinction between thresholded and non-thresholded compounds.

Study Types and Evaluation Principles

Studies in humans, epidemiological studies (see ► Chap. 19, "Epidemiological Methods in Regulatory Toxicology") would be the gold standard for the risk assessment of compounds to which humans are exposed. Those studies are mostly of observational nature with retrospective elements, and the data may be confounded by other risk factors (e.g., personal, behavioral, environmental characteristics, co-exposure to other agents) and background exposure. Therefore, specialized statistical and epidemiological methods are required to analyze these data for risk

assessment. It should be noted that the most valuable human data are often obtained from highly exposed populations (e.g., occupational cohorts) and do not cover dose ranges relevant for regulatory practice. Therefore, effects observed mainly at high doses are extrapolated to low doses. Opposite to human data with their high variability and heterogeneity exhibit data from studies of usually inbred strains of experimental animals in general very low heterogeneity and only moderate variability. Furthermore, confounding can be efficiently controlled by prospective and randomized designs. Therefore, animal studies have been considered also as gold standard for human risk assessment, even when two steps of extrapolation – from high to low doses and from animals to humans – are required. Extrapolation from high to low doses can be performed only within the animal species or within the human population.

Although the nature of statistical methods is general enough to be applied to both carcinogenic and noncarcinogenic data, the statistical methods for risk extrapolation must account for risk management principles used in regulatory practice, for example, the biologically based paradigm that genotoxic and/or directly DNA reactive carcinogens does not show a threshold exposure level below which no biological/toxicological effect is possible. Even when the existence of a threshold could be assumed for noncarcinogenic compounds or carcinogens which do not directly react with DNA, estimating that threshold dose is not straightforward, requires the use of statistical methods and is impaired by scarcity of dose–response data near the threshold. In most cases extrapolation from high to low doses and between species might be unavoidable.

A Road Map for Extrapolation

Risk extrapolation of both carcinogenic and noncarcinogenic compounds is preferably performed in a carefully planned investigation which should account for a number of critical check points listed in Table 1 as road map. Working through these points cannot be without considering the resources available for the assessment (e.g., available scientists and their profile of expertise, access to data, computational resources including software) and the time frame for delivering the low-dose extrapolation result. It should also be noted that this checklist may be applied iteratively for refining the assessment process.

Choice of Risk Parameters

The critical effects which define the risk parameter for extrapolation should have been identified at an early step of risk assessment, preferably *hazard identification*, in the form of adverse health effects which are potentially relevant for risk characterization and which can be assessed quantitatively for extrapolation from high to low doses. Quantitative extrapolation distinguishes three major

Table 1 Road map and checklist for extrapolation

1. **Data examination**: Screening and assessment of the available data on the compound, their quality, their relevance, and their suitability to construct dose–response relationships
a. When not only one but a whole class of compounds is assessed (e.g., dioxins), clarify how to proceed (lead compound, group risk index, toxic equivalence)
b. When more than one study and more than one endpoint are to be considered, check for pivotal studies and critical endpoints, respectively
2. **Risk parameters and measures**: Definition of risk parameters (the "risks") by appropriate choice(s) of critical effects and of the type of risk measures used for characterizing the risk and for which an extrapolation to low(er) doses is planned
3. **Dose–response relationship**: Construction and critical evaluation of the presence and of the type (linear versus nonlinear, monotone versus non-monotone, steepness at low and/or saturation at high doses) of the dose–response relationship. Check for availability of data from step 1 and discuss (risk-based) effect sizes regarding the choices made in step 2
4. **Assumption of a threshold dose**: Decision about the biological nature of the critical effect on the basis of all available biological data (structure-activity relationship, in vitro and in vivo tests, short- and long-term animal studies, epidemiological studies). Assess available evidence for the existence of a threshold dose only when the substance is not a genotoxic carcinogen
5. **Extrapolation**: Determination of the data suitable data for a fitting dose–response models and choice of a set of models or model classes which may fit the regulatory purpose
a. Derive in a first step a *point of departure* (PoD) *or reference point* (RP) from the available dose–response data and assess its statistical quality (e.g., central estimate vs confidence bound) and the degree of extrapolation, i.e., location related to observed dose range
b. Establish in a second step a health-based guidance value (HBGV), e.g., an acceptable/tolerable daily intake value (ADI/TDI), or characterize the gap between the PoD/RP and the estimate of current human exposure, e.g., through a margin of safety or margin of exposure (MoE)
6. **Outcome assessment**: Critical evaluation of the uncertainty of the regulatory value established in step 5 (e.g., by means of probabilistic methods), determination of data gaps, and formulation of recommendations of further research and additional data if appropriate

statistical classes of data types which express increasing statistical (not necessarily biological) content of information:

- Quantal (e.g., the occurrence of a defined illness)
- Categorical-ordinal (e.g., severity of allergies)
- Continuous (e.g., concentration of a liver enzyme or biomarker such as beta-2-microglobulin for renal tubular effects, often also denoted as quantitative data)

Carcinogenic effects seen in animal studies usually fall into the class of *quantal data*, with the occurrence of cancer (*cancer incidence*) or death from cancer (*cancer mortality*) as the relevant endpoints for human cancer risk assessment. Incidence and mortality are still the most relevant indices for cancer risk assessment and the control of cancer diseases in a population. For time-to-tumor or genomic alterations, both the biological database and the statistical tools available for dose-response analysis are still not well developed. In contrast to the evaluation of quantal data of carcinogenic effects where a standard suite of approaches exists (USEPA 2005; Working group on risk derivation within the subcommittee "Gefahrstoffbewertung" of the Committee

on Hazardous Substances 2005) the assessment of noncarcinogenic effects has been very diverse, in particular, regarding the specification of the relevant adverse events/effects and the identification of suitable parameters which describe effects best (Dourson et al. 2013). On the other hand, the database for noncarcinogenic endpoints is often larger and rich of *continuous data* which allow powerful dose–response analysis with smaller numbers of subjects than those needed for the quantal carcinogenic data. Data of the type *categorical-ordinal* were so far rarely analyzed for extrapolation purposes and require specialized analysis methods.

Choice of Risk Measures

When critical adverse health effects (disease incidence or the change of a continuous quantitative marker) are identified a quantitative risk measure R is defined, that describes the risk as a mathematical function $R(d)$ of the exposure dose d. In animal experiments the dose is usually expressed in units of mg/kg body weight administered per day. Alternatively, one may define the risk measure also in terms of the concentration of the substance, for example, as compound concentration in drinking water or in animal feed. In contrast to those external doses are concentrations of the compound in a target organ (e.g., blood, liver, kidney) considered as internal dose (see ▶ Chap. 29, "Toxicokinetic Models").

In the case of quantal data, $R(d)$ expresses the probability of the occurrence of the critical effect in the subject of investigation exposed to dose d:

$$R(d) = P(Effect|Dose = d). \tag{1}$$

The symbol P stands for *probability* (unfortunately, sometimes also denoted as risk). For many compounds one cannot neglect existing background exposure, either from exogenous or endogenous origin that adds to the total exposure (total exposure = background exposure + exposure through administered dose = d). Denoting the risk due to background by $R_0 = R(0)$, one may distinguish between additional and extra risk:

- Additional/added risk (above background): $R^*_{Add} = R(d) - R_0$
- Extra risk (of the substance): $R^*_{Extra} = \frac{R(d)-R_0}{1-R_0}$.

Risk measures for continuous data where $R(d)$ simply represents the effect size associated with the toxic compound can be defined accordingly as:

- Additional effect: $R^*_{Add} = R(d) - R_0$
- Relative effect (size): $R^*_{Relative} = \frac{R(d)-R_0}{R_0}$.

In quantitative risk assessments of environmental contaminants, in particular, when chronic inhalation exposure is assessed in epidemiological studies on cancer

Table 2 Estimates for unit risks (UR) based on LAI (2004) and unit doses calculated therefrom

Pollutant	UR[a] per μg/m^3	UD (1 μg/1 m^3)
Arsenic[b]	4×10^{-3}	2.5 ng/m^3
Asbestos[c]	2×10^{-5}	50 F/m^3
Benzene	9×10^{-6}	1.1 μg/m^3
Cadmium[d]	1.2×10^{-2}	0.83 ng/m^3
PAH (benzo(a)pyren)	7×10^{-2}	0.14 ng/m^3
2,3,7,8-TCDD	1.4	7.1 pg/m^3

[a]Estimated cancer risk for a person under constant inhalation exposure to a concentration of 1 μg pollutant per cubic meter of air for 70 years
[b]Arsenic and its inorganic compounds
[c]Based on 100 F/m^3 (F fibers)
[d]Cadmium and its compounds

incidence or mortality, the *unit risk (UR)* has been used as an international agreed risk measure, defined as the extra risk when a constant concentration of the toxic compound of 1 μg/m^3 exists in the inhaled air. Formally, this can be written:

$$Unit\ \ risk = P\left(C|constant\ \ exposure\ \ 1\mathrm{g}/\mathrm{m}^3\right) - P(C|no\ \ exposure) \qquad (2)$$

where C represents the occurrence of the observed disease, for example, cancer. Similar as for the additional risk, the first term on the right describes the probability of disease due to the exposure (1 μg/m^3) and the second the probability due to background, that is, the risk in absence of the compound. *UR* is then the excess lifetime cancer risk from continuous lifetime exposure to an agent at a concentration of 1 μg/m^3 in air. $UR = 3 \times 10^{-6}$ per μg/L means that three excess cancer cases are expected to develop per 1000,000 people all exposed to the *unit dose* (*UD*), that is, the daily exposure for a lifetime to 1 μg of the substance in 1 m^3 in air; same when exposed to drinking water in units of 1 μg/L water or through food in units of 1 μg/kg food. UR is the preferred measure for comparing the carcinogenic potentials of different toxic compounds (see, e.g., Table 2). It should be noted that without additional specification, all these risk measures assume lifelong constant exposure to the substance, in the past often assuming life length of 70 years.

Dose Extrapolation

Extrapolating from an established dose–response relationship available for the dose range

$$\mathrm{D}_{\mathrm{Experimental}} : \mathrm{d}_{\mathrm{min}} < \mathrm{d} < \mathrm{d}_{\mathrm{max}} \qquad (3)$$

to a lower dose range

Table 3 Possible scenarios for extrapolation

	Assumptions concerning the biological nature of action	
Data source	No threshold	Threshold
Epidemiological studies	Dose–response model	Approximation of the threshold
	Extrapolation in the model	Determination of a PoD/RP and using safety factor for intra-species variation
Animal experiments	Dose–response model	Determination of a PoD/RP and using safety factors for intra-species variation and inter-species differences
	Extrapolation in the model	
	Extrapolation to humans	

$$D_{Extrapolation} : d_L < d < d_U, \quad \text{where} \quad d_U < d_{min} \tag{4}$$

should distinguish between low-dose extrapolation with or without assuming the existence of a threshold dose. This distinction has guided risk assessment early on (WHO 1999; Boobis et al. 2009), although the question of the existence of biological thresholds has hardly been unequivocally resolved for any compound. Mainly three arguments question the existence of thresholds in general:

- "*additivity-to-background*": Even small exposure can increase existing effect levels.
- "*heterogeneity in the population*": Variation in sensitivity due to interindividual differences is not consistent with the assumption of a fixed break-point.
- "*missing evidence from epidemiology*": Responses in humans can occur even at very low doses.

The discussion on the existence of a threshold dose should not be confused with the discussion of assuming *low-dose linearity* (NRC 2009; White et al. 2009). However, existence of low exposure linearity and the use of linear low-dose extrapolation for noncancer health effects have been questioned by Rhomberg et al. (2011), with arguments related to the three above. Low-dose linearity is not in the focus of this chapter nor is a discussion on the role of mode of action (see, e.g., Dourson et al. 2013). This section concentrates on the threshold concept as it has been introduced in regulatory toxicology as pragmatic mean. The concept may be applied even though lower doses may show a biological effect, as long as it is considered as irrelevant or is practically indistinguishable from background because of statistical variation and measurement error. An overview on possible extrapolation scenarios for human and animal data depending on the assumption on the existence of threshold doses is given in Table 3.

Risk Assessment Under the Threshold Dose Assumption

When assuming the existence of a threshold below which no biologically relevant effect of the compound can be expected, regulation may start off to estimate that biological threshold, say D*. That estimate should be as precise as possible and as close as possible to the "true" value (i.e., statistically unbiased). Accounting for the uncertainty of that estimate, a sufficiently large safety margin represented by a safety factor (SF) would establish an *intervention dose* (ID) below which no biologically significant effects would be expected:

$$\mathrm{ID} = \mathrm{D}^{*}/\mathrm{SF}, \tag{5}$$

also referred as reference dose (RfD), defined by WHO (1999) as the maximum dose without significant or appreciable adverse effect on human health.

Traditional estimation of D* identifies the largest applied experimental dose at which there is still sufficient evidence of no adverse health when assuming a monotone increasing dose–response relationship. Practically, this is pursued through statistical hypothesis testing of each dose group against the control group, stepwise, starting with the lowest dose until one finds the highest dose at which there is still no statistically significant difference of the effects compared with the control (significance usually defined by a P-value <0.05). Consequently, the next higher must show a statistically significant difference in such a test procedure. The highest dose with no statistically significant effect is then denoted NOAEL (no observed adverse effect level) and serves as estimate of the biological threshold D*. That NOAEL is then used as PoD/RP, Table 1 step 5a. When a NOAEL cannot be identified (e.g., when all doses tested were statistically significant different from the controls), the smallest dose that caused a statistically significant effect denoted LOAEL (lowest observed adverse effect level) serves as PoD/RP. Since the LOAEL would in general overestimate D*, a higher safety factor (usually by a factor of ten higher) is used in that case. It should be noted that the estimation of the NOAEL may be significantly above or below the true threshold D*. The use of the NOAEL has been criticized therefore (EFSA 2017), predominantly for three reasons:

- *Strongly depending on the number of cases tested per dose group*. The larger the number of the examined subjects per dose, the higher is the statistical sensitivity (power) of the testing approach and thus the chance that a statistically significant effect is found at a dose. In converse, the smaller the sample sizes have been chosen per dose group, the higher will be the NOAEL, eventually higher than the highest dose tested.
- *Depending on the sensitivity of the biological assay.* The higher the sensitivity of the experimental determination of the biological effect, the smaller will be the NOAEL.
- *Strongly depending on the choice of doses and dose range*. The selection of the doses in $\mathrm{D}_{\mathrm{Experimental}}$ is crucial for the identification and localization of the NOAEL. If doses are widely spread in relation to true range where the

dose–response curve increases, the NOAEL can be determined only very vaguely and can be far above or below D*.

Safety factors (SFs) are applied in the second step of the establishment of the PoD/RP, for example, by dividing the NOAEL by SFs representing different types of uncertainty. Therefore, published literature and regulatory agencies have used the term uncertainty factors for SFs (see ▶ Chap. 26, "Benchmark Dose Approach in Regulatory Toxicology"). Traditionally, two types of SFs have been used (cf. Edler et al. 2002) when extrapolating from animals to humans (Table 3):

- $SF_{interspec} = 10$ to take into account the interspecies variability between animals and humans. It allows for the possibility that the average exposed person is up to ten-fold more sensitive than the average exposed animal for which the NOAEL was derived.
- $SF_{intraspec} = 10$ to take into account the interindividual variability. This is to ensure that a ten-fold more sensitive individual than that for which the PoD/RP value was derived will still be protected by the PoD/RP.

For a refinement of these SFs accounting for toxicokinetic and toxicodynamic data, see, for example, Dorne and Renwick (2005). It should be noted that even then these SFs are default factors not accounting for specific toxicokinetic and toxicodynamic knowledge of the toxic compound. A biologically based extrapolation would transform the dose–response relationship from animals to humans using toxicokinetic information by applying physiologically based toxicokinetic (PBTK) models, one for the animal strain and another for humans permitting the calculation of concentrations in target organs. A precondition, however, is that sufficient biological information is available to construct both PBTK models. For the situation in toxicodynamic modeling see ▶ Chap. 28, "Toxicodynamic Models."

If based on an animal experiment, dose has been converted from animal experiments to humans using interspecies extrapolation (USEPA 2005; ECHA 2012). For extrapolation from animals to humans, an allometric scaling is used where the administered doses are adjusted with body weights to the power of ¾ = 0.75.

Risk Assessment Without Threshold Dose Assumption

For compounds for which no threshold dose is assumed (non-thresholded), there are basically two approaches (see Fig. 1). At first, one can expand the dose range $D_{Experimental}$ to the entire dose range ranging from the "zero dose" (i.e., where only background exposure may exert an effect) to a maximum dose d_{max}, a practical limit beyond which an exposure is unrealistic. This dose interval D: $0 \leq d \leq d_{max}$ serves then as base of the dose–response assessment and risk estimation. However, this implies for the extrapolation to bridge several orders of magnitude, both in terms of response and in terms of dose. Although mathematical dose–response models can fit any dose range the biological database may not support such a wide range and the

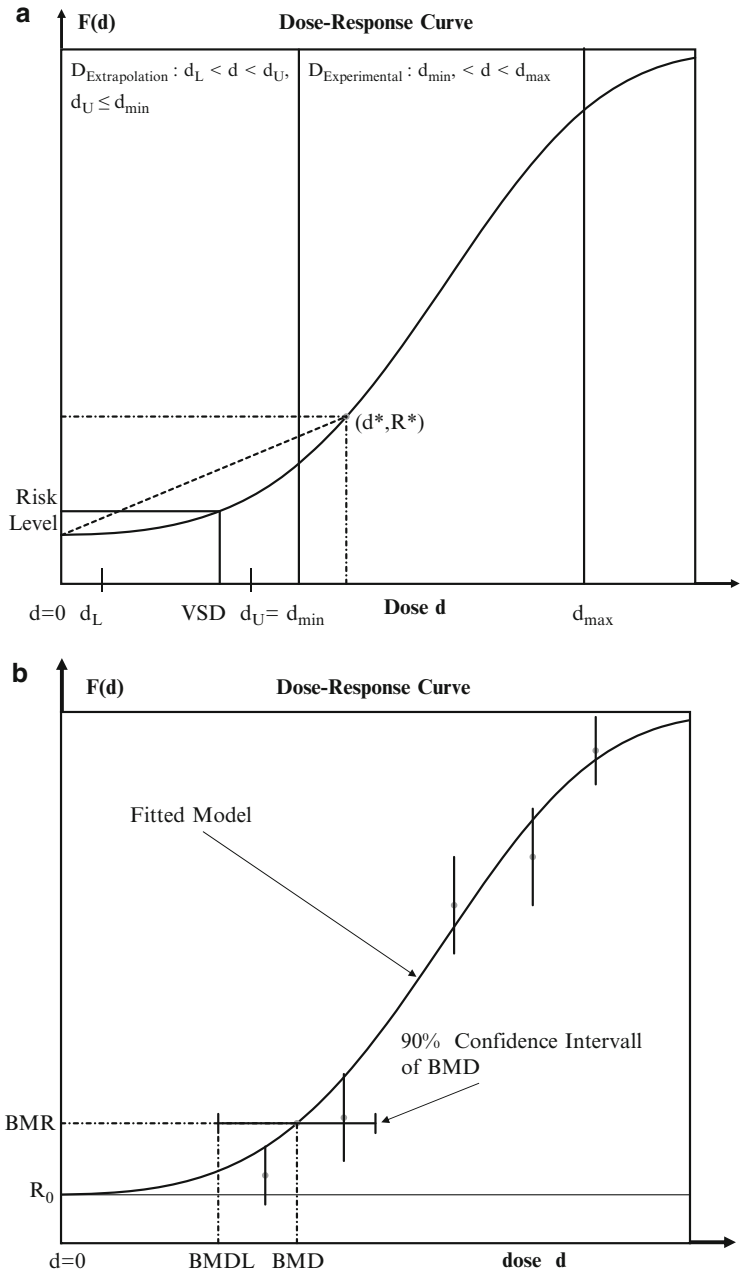

Fig. 1 (**a**) Dose–response curve $F(d)$ in the observed range $d_{\min} < d < d_{\max}$ and in the extrapolation range $0 < d < d_{\min.}$ (**b**) Benchmark dose (BMD) approach restricted to a left truncated dose range combining that $D_{\text{Experimental}}$: $d_{\min}$, $< d < d_{\max}$ and a limited extrapolation range $D_{\text{Extrapolation}}$ of the dose–response curve (copyright Annette Kopp-Schneider: the author thanks for providing the figure)

dose–response relationship *F(d)* fitted in the experimental range would only provide limited information on the relationship in the entire interval $0 \leq d \leq d_{\max}$ or the extrapolation range $D_{\text{Extrapolation}}$. It was found that different mathematical models equally good fitting the data in $D_{\text{Experimental}}$ provided largely diverging risk estimates when extrapolated to the low-dose range of interest, differing by several orders of magnitude. When, for example, the one-hit model, the multistage model, and the empirical models derived from the Weibull distribution and the log-normal distribution would all fit the data, one would obtain increasingly differing risk estimates when going to lower doses, in the order of

$$One\ \ Hit < Multistage < Weibull < Lognormal, \tag{6}$$

when excess risk is considered (see Krewski and van Ryzin 1981). This strong dependence of the risk estimates on the models selected and lack of biological justification for using a particular model has significantly compromised the use of these (and also other) models for regulatory purposes.

An alternative approach focuses on modeling dose–response for doses from $D_{\text{Experimental}}$ allowing only a limited extrapolation to $D_{\text{Extrapolation}}$. The best investigated approach of this type is the benchmark dose (BMD) approach (EFSA 2017) described below. It should be noted that the NOAEL approach described in the previous section requires not the threshold dose assumption and can be applied as pragmatic rule when statistical dose–response modeling leads to inconclusive results or when critical adverse health effects are too complex for available modeling tools.

The Limit Risk

A limit risk R_{limit}, interpreted as lifetime risk or lifetime cancer risk (LCR), is the probability that the exposure will cause cancer (incidence type of risk) or death from cancer (mortality type of risk) within average lifetime.

A first version of the limit risk approach stems from the second half of the last century known as the "virtually safe dose" (VSD) concept. It was at that time developed in response to difficulties of risk management in complying with US Food, Drug, and Cosmetic Act in the context of the Delaney Clause and when food additives found to induce cancer at any dose level were finally banned. The VSD was defined as dose associated with one additional tumor per one million subjects through lifetime exposure in the belief that a low LCR of 10^{-6} would be acceptable for a population of several millions. Later on that level was alleviated to an LCR of 10^{-5}. It should be noted that in a population of size 100 million about 500,000 persons might be diagnosed with cancer every year (IARC 2008). An LCR of 10^{-5} would then result in 13 additional persons with cancer per year in that population exposed during whole lifetime assuming an average lifetime of 75 years. Setting the limit risk R_{limit} to an LCR of 10^{-6} would allow only 1.3 additional cancer case per year in that population, see SCCS (2012).

The risk management of WHO, EPA, and US OSHA recommended an LCR of 10^{-5} for carcinogenic compounds. ECHA (2012) stated that "based on experiences, cancer risk levels of 10^{-5} and 10^{-6} could be seen as indicative tolerable risk levels when setting DMELs (derived minimal effect levels) for workers and the general population, respectively." Higher risks up to $1/1000 = 0.001$ have been accepted in the regulation in the working environment. All these limit risks established for humans are much lower than the measurable risk in a test group of animals tested for carcinogenesis which is generally not smaller than $1/20 = 0.05$; in large experiments at best $1/50 = 0.02$.

The most extensively used model for calculating an LCR has been the so-called linearized multistage (LMS) model (USEPA 1986). Based on the multistage mutation model of Armitage and Doll (see ▶ Chap. 28, "Toxicodynamic Models") the LMS is in essence a linear approximation of the multistage carcinogenesis dose–response model. Prominent model parameter is the slope factor (SF) used by the USEPA (USEPA 2005) as convenient descriptor of cancer potency in EPA's Integrated Risk Information System (IRIS) (see http://www.epa.gov/iris/). The LMS model is also a member of the set of models recommended for the BMD by EPA (USEPA 2012).

PoD Based on Benchmark Dose

The benchmark dose (BMD) approach is a general method of fitting dose–response models applicable for any dose–response data and basically it is built up by five steps:

1. Specification of type of dose–response data
2. Specification of the Benchmark Response (BMR) level
3. Selection of candidate dose–response model(s) for model fitting the data
4. Identification of acceptable models for modeling
5. BMD modeling and calculation of the PoD.

The BMD approach aims at determining a PoD/RP on an empirically and objectively verifiable basis and is applicable for all four scenarios described in Table 3. The BMD-based PoD was introduced into regulatory practice as the benchmark lower confidence limit (BMDL) of the dose at which response above background would not exceed a defined level, the BMR (Fig. 1b). Therefore, at the modeling step (5) above one determines one or a family of models which fit the observed dose–response data best in terms of a chosen statistical criterion (goodness-of-fit measure). Form a best fitting model – or best fitting family of models – one obtains then a statistical estimate of the benchmark dose (BMD) as the dose level that is associated with the change in the response defined through the numerical value of the BMR.

Simultaneously, one calculates during statistical estimation the confidence interval (BMD-CI) of the BMD corresponding to a specific confidence level

which has been traditionally defined as 10%. The BMD-CI is then an interval (BMDL – BMDU) on the dose scale where BMDL is the lower and BMDU the upper confidence bound covering with probability 10% the BMD of the best fitting model(s) given the data. The lower confidence bound BMDL is then a limiting dose which allows a statistical conclusion: "the BMD is with probability 95% not smaller than this BMDL." Choosing the BMDL as PoD allows then to say: "With 95% probability is the adverse health effect at the dose equal to this PoD not larger than the chosen BMR."

Note that in this set up the BMD-CI and BMDL are not necessarily within $D_{\text{Experimental}}$ nor within $D_{\text{Extrapolation}}$. The size and location of the BMD-CI and the location of the BMDL are a function of the dose–response data and depend strongly on wealth and precision of the available toxicity data. Various approaches ranging from pragmatic proposals to model averaging and other Bayesian approaches have been developed and discussed, in particular, by regulatory agencies such as FAO and WHO, US EPA, and EFSA. The most appropriate use of the BMD approach in risk assessment has been a topic of intense and controversial discussion since its introduction to low-dose extrapolation in the early 1980s and numerous efforts on guidance were launched to position the approach in regulatory toxicology. For details and more recent guidance see ▶ Chap. 26, "Benchmark Dose Approach in Regulatory Toxicology") The most relevant issues are as follows:

- The BMD approach uses all available dose–response data from a study and fits a set of mathematical models. It accounts for the statistical variability of the dose–response data by calculating the confidence interval of the BMD ranging from the lower bound (the BMDL) to the upper bound (the BMDU).
- The lower one-sided confidence bound BMDL (e.g., the BMDL_{10} when setting BMR = 10%) accounts for the statistical uncertainty in the data (with the statistical certainty level of 95%) and is used as PoD/RP.
- The estimate of the BMD and therefore also the size of the PoD depend via the fitted model(s) strongly on the definition of the size of the BMR.
- General advice on how to define the BMR requests consideration of a low but measurable response level, reflecting an effect that is negligible or nonadverse.
- Choosing the BMR very low might well comply with that advice but it would normally result in an extrapolation outside the range of the observed data and could induce severe model dependence of the BMDL: different models might return drastically different BMD and BMDL values, such that the risk assessment would be driven by the models fitted to the data and not by the observed data.
- Choosing the BMR high enough to avoid extrapolation could easily violate the advice and question the aim of regulation to protect human health.
- For quantal data a BMR = 10% of extra risk over background has been set as a default level in literature and in guidance of regulatory agencies for quantal data such as tumor incidence in animal experiments.
- For continuous data a BMR = 5% of change relative to background was proposed. Default values are helpful for orientation but cannot circumvent a

thorough scientific analysis of biological and toxicological consequences of different choices.

- Suites of models were recommended and used in practice. When different models are fitted to the data and when some models fit equally well but result in different BMDs and BMDLs, selecting the BMDL of the best-fitting model is likely to underestimate the uncertainty in the BMD approach, while selecting the model with the lowest BMDL generally results in an overestimate of the risk.

Other PoDs

Depending on the dose–response data available, two other methods concur with the BMD approach in practice:

T25: Defined as the chronic dose with 25% of the animals with tumors (at a specific tissue site after correction for spontaneous incidence and within the standard lifetime) the T25 is likely within the range of the experimental data (Dybing et al. 1997). An adjusted T25 is obtained as

$$HT25 = T25/(bw_{human}/bw_{animal})^{0.25} \tag{7}$$

and an LCR can be calculated as

$$LCR = exposure\ \ dose/(HT25/0.25) \tag{8}$$

In contrast to BMD a T25 can be clearly identified even when besides the control group, only one dose group is available.

TD50: The TD50 value was introduced primarily for ranking of carcinogens in the Carcinogenic Potency Database (CPDB).

It characterizes the dose which, if administered chronically for the standard lifespan, will halve the probability of remaining tumor free (Sawyer et al. 1984; Gold et al. 1999). The determination of the TD50 value is complicated by intercurrent deaths due to causes other than tumorigenesis and the non-observability of the time of onset. The TD50 has been used as PoD when the toxic substance was administered chronically for the standard lifespan of the species, but is not recommended for low-dose extrapolation.

Margin of Exposure (MoE)

Risk assessment of compounds that are both genotoxic and carcinogenic presents particular difficulties, since the effects of such compounds are normally regarded as being without a threshold such that no safe level can be postulated. Therefore, low-dose extrapolation has been found inappropriate for genotoxic carcinogens. Pragmatic risk management approaches such as the application of the ALARA (As Low as Reasonably Achievable) and the TTC (Threshold of Toxicological

Concern), which establishes exposure thresholds for chemicals present in food, dependent on chemical structure, have been discussed and occasionally applied as possible rescue. However, such approaches cannot inform risk managers on urgency and extent of the risk reduction measures needed.

More recently the margin of exposure (MoE) approach has been applied by both the European Food Safety Authority (EFSA) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) not to bridge the gap between the PoD/RP and human exposure but to describe the extension of that gap (Barlow et al. 2006). The MoE is numerically defined as the ratio of the point of departure (PoD/RP) of the critical effect to the predicted or estimated exposure level (WHO 2009). The magnitude of the MoE gives an indication of the level of concern without extrapolation to the substantially lower exposure levels usually encountered in human situations: the larger the MoE, the smaller the potential risk through exposure. The MoE should, however, not be used for a numerical quantification of risk but stay as qualitative advice for risk management. Therefore, the MoE is not recommended for extrapolation and the calculation of actual risks in the exposed population (Barlow et al. 2006). However, an MoE of 10,000 or more, based on animal cancer bioassay data, would be considered as of low concern (EFSA 2005). An MoE higher than 10,000 based on $BMDL_{10}$ can, in cases of lifelong exposure, be associated with an LCR lower than 3.5×10^{-5} if based on a male rat experiment and lower than 7×10^{-5} if based on a male mice experiment and using linear extrapolation (ECHA 2012; USEPA 2005).

Cross-References

- ▶ Benchmark Dose Approach in Regulatory Toxicology
- ▶ Epidemiological Methods in Regulatory Toxicology
- ▶ Toxicodynamic Models
- ▶ Toxicokinetic Models

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Probabilistic Methods in Toxicology

24

Odile Mekel and Rainer Fehr

Contents

Abstract

The concepts of "variability" and "uncertainty" play a central role in exposure and risk assessment. Traditionally applied worst-case scenarios do not adequately reflect the requirements of modern practice. Methods of probabilistic analysis, such as Monte Carlo simulations, are promising developments for sound consideration of these aspects.

O. Mekel (✉)
Division Healthy Settings, NRW Centre for Health, Bochum, Germany
e-mail: odile.mekel@lzg.nrw.de

R. Fehr
Department of Public Health, University of Bielefeld, Bielefeld, Germany
e-mail: rainer.fehr@uni-bielefeld.de

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Background

Regardless of the topic in question, variability and uncertainty are aspects of modeling and assessing health risks which need to be taken into account (Bogen et al. 2009; Mekel and Fehr 2000; US-EPA 2011; Schendel et al. 2018). "Variability" refers to the (statistical) distribution of the studied phenomena, while "uncertainty" refers to those parameters, factors, and models which are lacking or incomplete. This chapter will expand upon the concept of uncertainty and variability, describe methods of probabilistic estimation and sensitivity analysis, and provide an overview of suitable software.

Variability refers to real heterogeneity with respect to space, time, or persons and represents a feature of the system studied. Subdividing sources of variability according to space, time, and population provides a useful means for their understanding. Examples of temporal variability include, for instance, seasonal food consumption patterns or patterns of activities varying on a weekly basis. Both small and wide-area variations are observed in environmental pollution. Examples of intraindividual variability concern behavioral and personal features (Table 1).

In practice, variability can be taken into account through subdividing the studied system into a number of subgroups which are then analyzed separately. In research design and classical statistics, this is called "stratification." The phenomenon of variability cannot be resolved by additional studies, these can serve solely to characterize the degree of variability more precisely. This results in a need for political–administrative decision-making on the desired level of safety in environmental policy.

Uncertainty, in contrast, is a researcher's feature. It results from incomplete or lacking knowledge on aspects of the studied system. Uncertainty, just as variability, contributes to variation of analytical results. Types of uncertainty include scenario, parameter, and model uncertainties. The former concerns, e.g., an exposure pathway which was overlooked. Parameter uncertainty can result from samples lacking representativeness. The third type of uncertainty regards the modeling quality, as, e.g., inclusion or exclusion of a relevant model parameter. In principle, uncertainty can be reduced by doing additional research (Table 2).

Table 1 Sources for variability. (Based on US-EPA 2011)

Category	Variability source concerning. . .	Examples
Time	Long-term resp. short-term variation	Concentration level
		Weather
		Dietary intake
		Seasonal variation
		Long-term trends
		Weekly interval of activity patterns
Space	Regional; small scale	Spatial variable concentration
		Regional dietary habits
Population	Interindividual variability	Personal characteristics: e.g., bodyweight, age
		Behavior: e.g., time budget, activity pattern

Table 2 Sources for uncertainty. (Based on US-EPA 2011)

Category	Uncertainty source concerning...	Examples
Scenario uncertainty	Descriptive errors	Incorrect or incomplete information
	Aggregation errors	Spatial and temporal approximations
	Judgments errors	Selection of a wrong model
	Incomplete analysis	Overlooking important exposure pathways
Parameter uncertainty	Measurement error	Imprecise or biased measurements
	Sample uncertainty	Small or nonrepresentative sample size
	Variability	In time, space, or activity
	Surrogate data	Chemicals with similar structure
Model uncertainty	Relation error	Incorrect conclusions from correlations
	Modeling error	Non-consideration of relevant parameter

Both phenomena, variability and uncertainty, are relevant to each step of risk assessment. Distinguishing between sources of variability and uncertainty is important regarding two aspects: Firstly, with respect to interpreting the results, when assessing toxicity, for instance, it is important to know which variability exists within the population in question. Additionally, the reliability of this toxicity assessment matters: How sure are we that the toxicity and its variability was estimated correctly?

Secondly, the distinction between variability and uncertainty is important for the following reason: While variability impacts on the assessment's precision and its generalizability, uncertainty can lead to incorrect statements.

Variability and uncertainty of variables often occur together. If certain aspects of variability are unknown and stratification therefore is not possible, this lack of knowledge contributes to the uncertainty of the analysis. The quantification of soil ingestion from mouthing behavior of small children can serve as an example: It is well-known that there are large differences between children concerning the daily soil ingestion. The study design and methods of most recent studies still leave many open questions. For instance, it is questionable to which extent the soil ingestion was determined correctly; what is the variance between children; which type of statistical distribution can best describe the variability; and how do seasonal factors influence these values.

Methods for Quantifying Variability and Uncertainty in Risk Assessment

Point Estimates

In traditional risk assessment, single values or point estimates are commonly being used for representing the input model variables. In order to describe the typical conditions, for model variables having an empirically describable variability, measures of central tendency, i.e., mean or median, are being used. Such an estimate is

referred to as "typical case." For the purpose of considering variability and uncertainty adequately, especially with respect to sufficient health protection, assumptions are mostly conservative or "unfavorable." So far, upper percentiles like 90th or 95th percentiles of variables or – if such measures were not available – the worst conceivable assumptions were used for exposure assessment. This results in the so-called worst-case approach. Worst-case assumptions are usually a combination of variability and uncertainty concerning model variables. It is problematic that worst-case estimates often do not describe realistic exposure situations.

Probabilistic Estimates

Probabilistic assessments make use of the entire distribution of all or several model variables (Cullen and Frey 1999). Simulated values are randomly chosen from these distributions according to their statistical parameters and then linked to other randomly chosen values according to the model's algorithms. An example of this principle using the "nutrition" pathway in probabilistic exposure assessment is illustrated in Fig. 1. From the distribution of each of the three input variables, randomly chosen simulation values are being selected, e.g., 1.14 kg/day for food consumption, 4.9 ng/kg for pollutant concentration, and 9.7 kg for body weight. According to the model equation, the resulting exposure is 0.58 µg/kg body weight-day. This procedure is repeated through Monte Carlo simulation many times. The results of these simulations, in turn, can be displayed in a distribution, too. This distribution then represents the exposure assessment's results and can be described by its statistical parameters such as mean, standard deviation, and percentiles.

By using entire distributions for estimation, each possible feature of a variable, including the "tails" of the distribution, is combined with other model variables according to its respective probability. This results in better insights about the populations' exposure and more meaningful information regarding the spread and confidence interval of the calculated exposure or risk. Additionally, probabilistic methods provide the possibility to include all available information into the assessment, as opposed to an arbitrary selection of percentiles.

Sensitivity Analysis

By conducting sensitivity analysis, model variables that contribute most to the spread of the results can be isolated (WHO/IPCS 2008): If, e.g., the distribution of input variables that are identified as being influential to the final results relies on sound data, the estimation can be considered sound. Body weight, for instance, could have strong influence on the final results. If the probability distribution of body weight applied is based on a representative population sample, the calculated variation can be considered reliable. If, in contrast, the input variables that are identified as influential to the final results rely on a relatively weak data basis, the results, correspondingly, are unreliable. Such findings can also point at further need

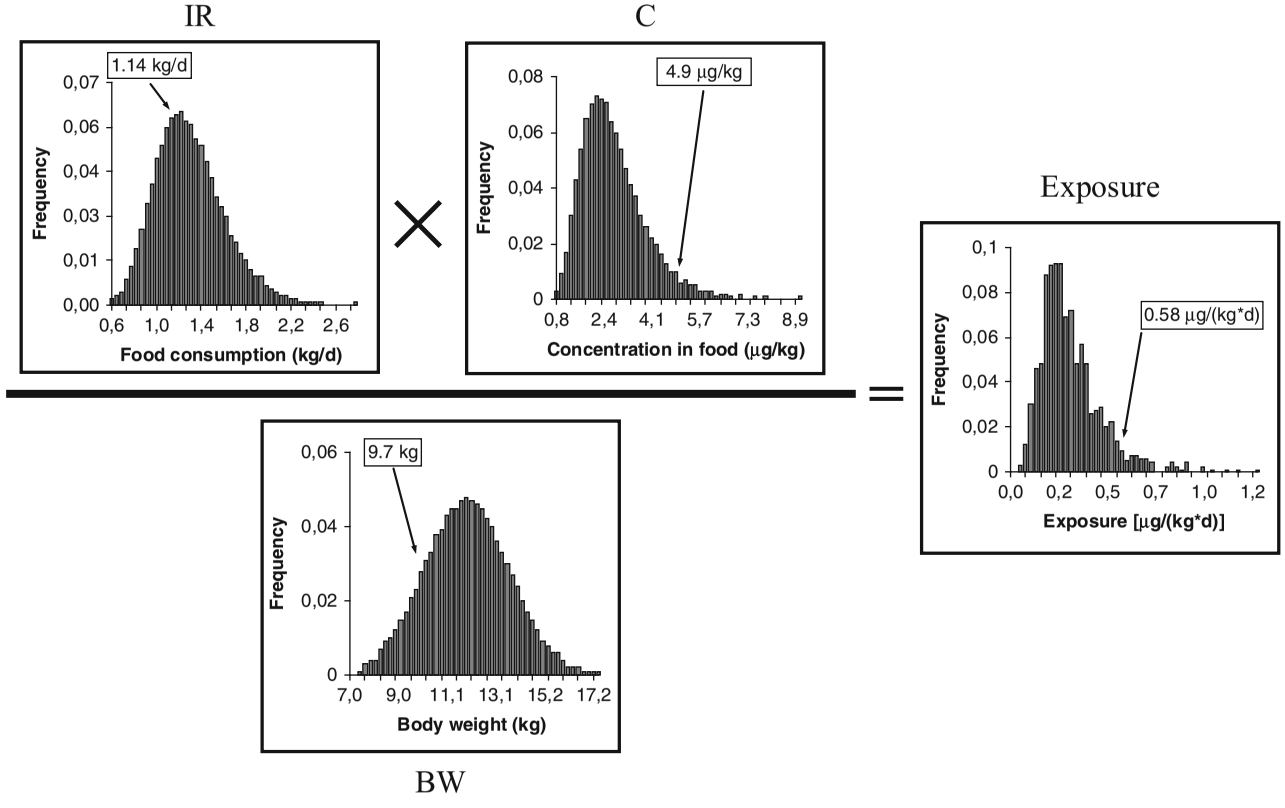

Fig. 1 Exemplification of a Monte Carlo simulation for the example of dietary exposure of children (*IR* = food consumption [kg/day], *C* = concentration in food [µg/kg], *BW* = body weight [kg])

for research regarding that variable. From this background, variables, which rely on a weak data basis but are identified as not significantly impacting the final results, will not necessarily require an effort to improve the data basis.

The simplest type of sensitivity analyses are *What-if*-analyses: The size of each input variable is modified (e.g., in steps of 10%), respectively, while the other variables are kept constant, studying the respective influence on the final result. Itemizing for input values, the most sensitive variables can be identified.

Meaningful sensitivity analysis requires data on variation that usually cannot be obtained from point estimates, but are easily available from probability distributions. Sensitivity analysis is not meaningful when using worst-case point estimates, because the maximum value is used for several input variables (e.g., 100% resorption). The combination of probabilistic estimates and sensitivity analysis provides information about the reliability of the estimates and its possible consequences regarding risk management.

Application Potential in Dose–Response Assessment

Research and development in the area of probabilistic modeling so far have focused on exposure assessment (Mekel et al. 2007; Lindtner 2014). In recent years only, efforts were made to investigate their application potential in dose–response assessment as an alternative or addition to the application of so-called uncertainty factors that traditionally have been used when transferring data from animal studies to humans. In the Netherlands, these methods are applied in parallel to traditional, deterministic risk assessment of new and existing chemicals and pesticides (Vermeire et al. 2001). Similar developments can be observed in other countries (Chiu et al. 2018) but often have not become part of regulatory practice yet.

Software for Probabilistic Exposure and Risk Assessment

Faster computers have enabled the application of computationally intensive probabilistic modeling in recent years. Specific commercial software tools for conducting probabilistic simulations are available. These software tools are not specifically designed for use in areas like toxicology or environmental health, but are used in a variety of disciplines where risk and decision analysis is an issue, in particular, in areas like economy and finance.

For performing a probabilistic exposure and risk assessment, the two most popular commercial systems are @Risk (http://www.palisade.com) and Crystal Ball (http://www.oracle.com). Both systems work directly as add-ins for spreadsheet software like Excel. @Risk is now available in seven different languages.

Both systems work in similar ways: Both require (i) a user-defined model to be implemented in a spreadsheet and (ii) the specification of the probability distributions for the model input variables. Differences exist in performance, e.g., in terms of clarity, provision of (partly) automatic functions, graphs, etc. Both systems offer a

large amount of different options for performing probabilistic analysis, necessitating, however, considerable intensity of training. Standard statistical software like SAS, SPSS, or R can be used for probabilistic assessment, too, but all simulation steps need to be programmed. Again, this requires extensive knowledge of the statistical packages.

Cross-References

- ▶ Computer-Based Prediction Models in Regulatory Toxicology
- ▶ Dose-Response Analysis: Identification of Threshold Levels for Chemicals
- ▶ Dose–Response Relationship and Extrapolation in Toxicology: Mechanistic and Statistical Considerations
- ▶ Exposure Scenarios in Toxicology
- ▶ Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds
- ▶ Read-Across Methodology in Toxicological Risk Assessment
- ▶ Risk Characterization in Regulatory Toxicology
- ▶ Risk Communication: Challenges for Toxicologists and Other Risk Experts
- ▶ Statistical Evaluation Methods in Toxicology
- ▶ Toxicological Risk Assessment
- ▶ Uncertainty Analysis in Exposure Assessment-Relevance for Toxicological Risk Assessment

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Exposure Analysis for Indoor Contaminants 25

Gerhard Volland

Contents

Abstract

Due to changes in living and working habits, most individuals spend about 80–90% of their time in public and private indoor environments. Those offer a broad diversity of pollution situations. Several hundred of very volatile, volatile, semi-volatile, and particular organic matter (VVOCs, VOCs, SVOCs, and POMs) can be detected in indoor air. Emitting from construction materials (e.g., floorings, paints, furniture, joints), consumer products (electrical and electronic devices), as well as cleaning products, they are one of the determining factors for indoor air quality (IAQ). The wide variety of pollutants, exposure levels, differences in sensitivity, as well as different cultural habits and ways of living

G. Volland (✉)
Material Testing Institute (MPA)-Otto-Graf-Institute Pfaffenwaldring, Schwäbisch Gmünd, Germany

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complicate the assessment of risk. In a variety of reports, indoor air pollutants have been associated with health problems. Therefore, it must be of special interest to obtain reliable data to assess the IAQ. The basis for reliable and comparable data is given by a series of international and national standards for the sampling and determination of volatile (VOC) and semi-volatile (SVOC) organic compounds in indoor air.

Keywords

Indoor air pollution · Organic pollutants · Sampling and determination strategies

Objectives and Purpose for the Determination of Volatile and Semi-volatile Organic Compounds in Indoor Air

Indoor air, as dynamic system, is generally characterized by a wide variety of organic pollutants in differing concentrations (Salthammer 1999; Uhde and Salthammer 2007; Edwards et al. 2005; Schleibinger et al. 2001; Hofmann and Plieninger 2008). **Measurements of indoor air pollution are carried out for different reasons.** However in most cases, occupants **complain about poor air quality**, which often is correlated with complaints about odor and/or unspecific health problems such as headache, sleeplessness, lack of concentration, and fatigue (WHO 2000, 2010; COMEAP 2004; Anses 2011; EPA). Based on the results of a first measurement or a previous survey of a building, it might be necessary to **determine if a specified limit or guideline value is maintained**. Last but not least, the effectiveness of a remedial treatment has to be proven. A special task is to correlate observed or suspected effects on occupant health with indoor pollution. Due to these different questions, **individual sampling and determination strategies have to be applied**. Complaints from occupants in public buildings (e.g., offices, schools, and kindergartens) are often characterized/accompanied by the presence of different complaints and differing health problems. As normally, only few information on possible pollutants and their sources exists, these cases regularly require an extended search for the possible causes of the complaints. In general, it is advisable to use questionnaires to obtain a systematic record of complaints as well as a systematic record of the affected building (e.g., ISO/DIS 16000-32 – Investigations of constructions and pollutants and other injurious factors – inspection). Based on the obtained information, an individual sampling strategy has to be developed. In the ISO Guides 16000-1 (general aspects) and 16000-5 (volatile organic compounds – VOCs), the general rules for different sampling strategies are specified. In addition to these general aspects, ISO Guides 1600-2 and 1600-12 describe the sampling strategies for formaldehyde and polychlorinated biphenyls and dibenzo-dioxins/furans (PCB, PCDD/F), respectively. In cases of complaints about "bad" indoor air, it is often useful to know the average level and the range of the concentration of organic pollutants in indoor air (Krause et al. 1991; Schleibinger et al. 2001; Hofmann and Plieninger 2008).

Table 1 Development of the average concentrations of typical volatile organic compounds in indoor air (median) within the period from 1985 to 2008 in indoor air in Germany

Compound	1985/1986 (Krause et al. 1991)	1999 (Schleibinger et al. 2001)	2006 (Hofmann and Plieninger 2008)
	Median in µg/m^3		
Toluene	62	28	12
Sum of C9 aromatic hydrocarbons	23	8	10
1-Butanol	<1	27	11
Limonene	13	8	6
Formaldehyde	55	38	32
Hexanal	<1	34	21

In this context, it must be noted that due to regulations and technical progress, new building and consumer products have been implemented on the market. These products are in general characterized by lower emission rates and the substitution of critical ingredients, e.g., solvents. This influences the average composition of indoor air. The reduction of the average concentration of aromatic hydrocarbons in indoor air is an example for this development (see Table 1).

Based on first investigations, often the question occurs whether threshold or guideline values are exceeded in the indoor air. Besides formaldehyde, **most threshold or guideline values for VOC and SVOC are long-term guidelines**, regarding an **average concentration** in indoor air over a longer period (e.g., annual average). Depending on the definition of the guideline value, short-term sampling and/or long-term sampling methods have to be applied. Guideline values for indoor air quality are published by the World Health Organization (WHO 2000, 2010), for the United States (EPA 2007; OEHHA 2012), France (Anses 2011), the United Kingdom (COMEAP 2004), and Germany (Umweltbundesamt 2007). Table 2 gives an overview of existing guideline values in different countries.

Screening Methods

Besides the given low concentrations of organic pollutants in indoor air, which normally prevents the use of screening methods, these methods just detected a "sum" of volatile compounds in indoor air. In principle, gas chromatography with flame ionization detector (FID), photo-ionization detector (PID), and photo-acoustic sensor (PAS) is applicable for screening methods. These methods may give a quick overview of possible indoor air pollution. In practice, however, these results often show substantial deviations from the real concentration of VOC in indoor air. Generally not suitable are commercially available short-term tubes used for air examination at the workplace in the range of workplace-related limit values. For special indoor air pollutants, e.g., formaldehyde, commercial enzyme-based screening systems (e.g., "Bio-Check") are available and suitable for pretesting.

Table 2 Indoor air guideline values for selected indoor air pollutants in different countries compared with the median in indoor air in Germany

Organic compound	Median (P50) indoor air Germany (Hofmann and Plieninger 2008)	WHO (2010)	USA (chRELs) (EPA 2007; OEHHA 2012)	Germany (recommended concentration RW I) (UBA 2007)	France (Anses 2011)	UK (COMEAP 2004)
	Concentration in µg/m^3					
Formaldehyde	32.5	100 (30 min)	< 20 (EPA) 9 (OEHHA)	120 (30 min)	10	100 (30 min)
Naphthalene	1	10	9	2	–	–
Toluene	1	–	300	300	–	–
Trichloroethene	1	–	600	–	20	–
Tetrachloroethene	1	250	–	–	250	–

Sampling

Generally, the sampling strategy has to be adapted to the individual case. Sampling of indoor air should be carried out **at room temperatures between 19 °C and 24 °C** and a **relative humidity in the range of 30% and 70%** (comfort level see ISO 7730). Besides that, other **important parameters** like the **ventilation**, the nature of the **sources**, and the type of indoor environment have to be paid attention in choosing the conditions for sampling indoor air. Two basic sampling systems are applicable for the determination of VOC and SVOC in indoor air. Active short-term sampling is characterized by drawing the air through defined absorption systems, e.g., charcoal, polyurethane foam, or different sorts of silica gel.

The short-term sampling strategy for formaldehyde and VOC, for example, in natural ventilated rooms, demands intensive ventilation as a first step. After this, ventilation doors and windows have to be closed for about 8 h (preferably overnight). The sampling starts after this period (preferably next morning) without further ventilation (see ISO 16000-2 and ISO 16000-5). The sampling volume varies according to sampling method and the interesting compound between 1 and 10 l (thermal desorption and gas chromatography/mass spectrometry) up to 400 m^3 (determination of dioxins and furans). The hourly sampling rate should be less than 10% of the room volume or less than 10% of the ventilation rate (see ISO 16000-1). The disadvantage of the short-term sampling technique is that the result only represents the composition of indoor air during the sampling time. Normal differences in indoor air, effected by different air exchange rates or temperature-influenced changes in the emission rate, are not detected by active sampling methods. To detect these influences, a series of sampling are necessary.

To determine the average concentration of an indoor air pollutant, long-term sampling strategies should to be applied. If passive sampling systems for the interesting pollutants are available, this method offers the chance to determine the average indoor air concentration. Passive sampling systems are available and tested for aldehydes (see ISO 16000-4) and VOC (see ISO 16017-2 and EN 14412). For long-term measurements, generally no preconditioning is necessary. Sampling is carried out under actual living conditions. Occupants maintain their usual ventilation habits. It is recommendable to request and document these habits before the sampling starts. Deviations during the sampling period should be documented as well. For long-term measurements in offices, schools, and kindergartens, it is recommendable to determine the room temperature, the humidity, and especially the concentration of CO_2 continuously parallel to the sampling. Disadvantage of the passive sampling method is that concentration peaks and concentration gradients are not detectable. Figure 1 gives an example of the results for toluene in indoor air in different indoor environments obtained by active short-term and passive long-term sampling method (Bruno et al. 2008).

Aspects of the sampling and measurement strategy as well as examples and limitations of the different sampling techniques are given in the ISO standards 16000-1, 16000-2, 16000-5, and 16000-12 and ISO 16017-1 and 16017-2.

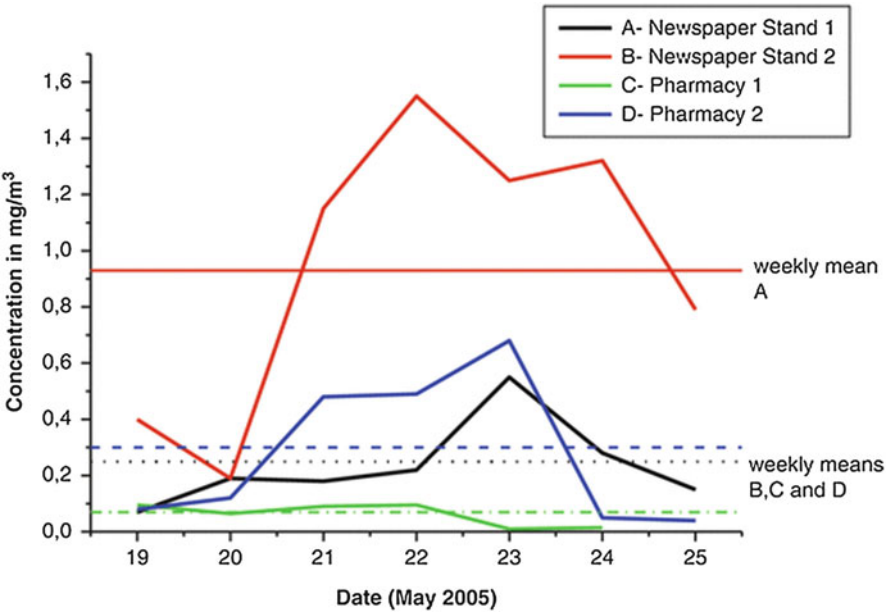

Fig. 1 Weekly trend of toluene concentration in various indoor environments (Bruno et al. 2008)

Determination of Organic Pollutants in Indoor Air

After sampling volatile and semi-volatile compounds in indoor air on solid-phase sorbents, several detection methods are applicable. For **VOC and SVOC, chromatography/mass spectrometry (GC/MS low and high resolution)** combined with either thermal desorption or solvent extraction is an appropriate method. ISO 16017-1 gives an overview of suitable sorbents tubes and the related accuracy. For common concentrations of VOC in indoor air, the standard deviation using thermal desorption and GC/MS is in the range of 1–5% (see ISO 16017-1). Using charcoal sorbent tubes combined with solvent extraction, comparable standard deviation can be obtained (see VDI 2001-2). For semi-volatile organic compounds like biocides or polychlorinated biphenyls, absorbed on polyurethane foam (PUF), standard deviations in the range between 10% and 25% are frequent (see Table 3).

Formaldehyde and other **carbonyl compounds (aldehydes and ketones)** can be absorbed on **silica gel cartridges coated with 2,4-dinitrophenylhydrazine (DNPH).** The formed DNPH derivatives are analyzed utilizing high-performance liquid chromatography (see ISO 16000-3). The average random error (standard deviation for duplicate samples) for the determination of formaldehyde in indoor air is about 12% (see ISO 16000-3). This proves that the available methods for sampling and detection of organic compounds in indoor provide sufficiently accurate results:

Table 3 Selected dioxin-like PCB – an example for the accuracy of the determination of toxic semi-volatile organic compounds in indoor air (Volland 2006)

	Laboratory A		Laboratory B
	Sorbent PUF method EPA TO-4A GC/MS (HR)		Sorbent PUF method ISO 16000–14 GC/MS (LR)
PCB congener	Arithmetic mean ($n = 4$)	Standard deviation (abs.)	Separate operation
	Concentration in ng/m^3		
PCB 77	0.68	0.05	0.67
PCB 118	12.7	1.52	17.00
PCB 156	2.60	0.14	2.46
PCB 167	1.0	0.11	1.34
WHO-TE (max.) in pg/m^3	5.0	0.59	5.4

Detailed information for the determination of VOC and SVOC is given with ISO 16000-3, -4, -6, -13, -14, -31; ISO 16017-1 and -2; EN 14412; VDI 2100; VDI 2464, VDI 3498 and EPA Compendium Methods for the Determination of Air pollutants in indoor air and EPA Compendium Methods for the Determination of Toxic Organic Compounds in Ambient Air (EPA 1990, 1999).

The detection sensitivity of the applied methods ranges from few pg/m^3 (e.g., PCDD/F method ISO 16000-14) to several hundred micrograms/m^3 for the detection of organic pollutants.

Limits and Errors of the Determination of Organic Indoor Air Pollutants

Despite existing guidelines and standards for sampling and determination of organic pollutants in indoor air, very often, differing results for the same building are reported. Indoor air is a dynamic system, and thus, the concentration of organic pollutants in indoor air is influenced by a set of parameters. Besides the wide range of the contamination within a building (see Table 4), those differences are mainly caused by three parameters.

Strength and emission characteristic of the source: Depending on the room temperature, the temperature of the building element, and the air humidity, the emission characteristics of the sources change.

Ventilation: In most cases, the concentration of volatile organic pollutants in ambient air is significantly lower than in indoor air. Thus, different ventilation rates influence the obtained results. Depending on the tightness of a building envelope, the wind speed influences the ventilation even if the windows are closed.

Absorbing effects and secondary sources: Depending on the vapor pressure, organic compounds can absorb at the surfaces of, e.g., walls, floors, as well as on dust particles (see Table 3). In particular, SVOCs generate relevant secondary sources based on absorbing effects.

Table 4 Examples of the concentration range of phthalate-based softeners [Di-ethylhexyl-phthalate (DEHP)] and organophosphorous-based flame retardants [Tris-chloro-ethylphosphate (TCEP)] in indoor air and house dust in German schools (Hansen et al. 2001; Volland et al. 2010)

	Living area and sleeping rooms	Classrooms	Community area (e.g., dining room)
Softener (DEHP) (Volland et al. 2010)			
House dust	Range and arithmetic mean (Ø) in mg/kg		
Boarding school A	300–2,300	500–2,600	<50
	Ø 1,200	Ø 1,880	
Boarding school B	110–740	350–575	<50
	Ø 400	Ø 450	
Indoor air	Range and arithmetic mean (Ø) in µg/m^3		
Boarding school A	<0.1–0.57	n.d.	<0.1
Boarding school B	0.1	0.1	<0.1
Flame retardant (TCEP) (Hansen et al. 2001)			
House dust		Range in mg/kg	
School C	Nonexistent in this building	320–530	Nonexistent in this building
School D		770–2,190	
School E		410–1,450	
Indoor air in µg/m^3		Range in µg/m^3	
School C	Nonexistent in this building	0.36–0.43	Nonexistent in this building
School D		1.2–3.9	
School E		0.3–2.0	

Influence of Emission Characteristics and Ventilation

The **emission rate** of VOC and SVOC is commonly determined by the diffusion potential and the **vapor pressure** of the organic compound. Figure 2 points out how different vapor pressures resp. boiling points of solvents in coatings influence the emission characteristics of volatile compounds (Zellweger et al. 1997). In general, VOC with boiling points between 60 °C and 150 °C will lead to short and high concentration of VOC in indoor air immediately after application of the product. The emission of products containing organic compounds (e.g., solvents) with high boiling points (>180 °C) resp. low vapor pressure is characterized by long-term emission of those compounds combined with a low concentration in indoor air. Regarding the influence of the temperature, it is obvious that **higher temperatures** will **increase the emission rate**. The concentration of dioxin-like PCB in indoor air in buildings with PCB-coated ceiling slabs, for example, increases from 3.5 up to 13.6 pg WHO/TE/m^3 (Volland et al. 2006). At the first glance, normally, the room temperature is taken in account. Due to **structural conditions of a building**, parts of this building may have **different temperatures**. Depending on the season and the

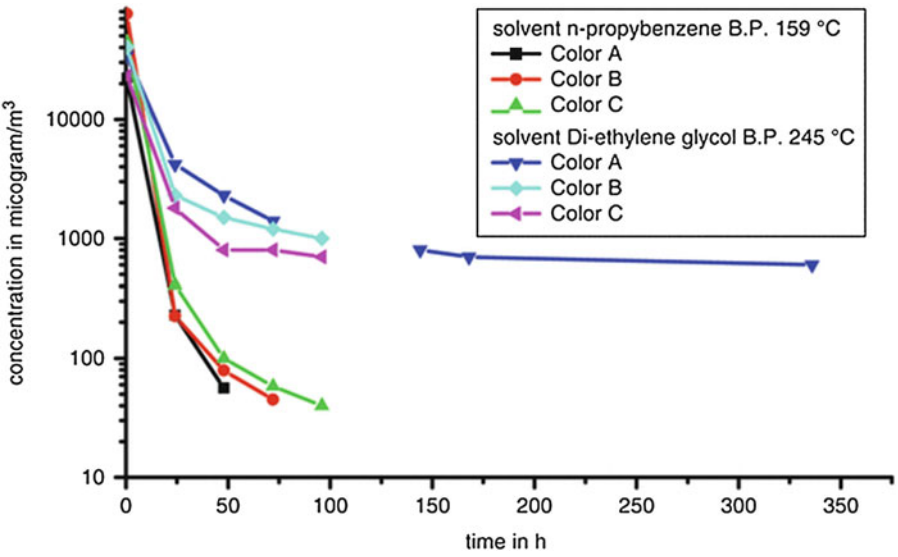

Fig. 2 Development of the concentration of n-propyl-benzene and diethylene glycol in test chamber air within the period after application of different products (Zellweger et al. 1997)

actual weather conditions, the temperature of these building materials ranges from 10 °C to 60 °C. Longer periods of sunshine, for example, will increase the temperature of building components. Experience shows that this effect is relevant for components containing indoor air pollutants. Influences caused by different temperatures of the building itself can be shown, regarding the season of sampling. Figure 3 gives an example of the influence of the room temperature and the season, when sampling was conducted.

Depending on the definition of a long-term guideline value, the interesting average concentration of indoor air pollutants also depends on the ventilation. The effects of sampling carried out according to ISO 16000-1 (see short-term sampling above) or when sampling is carried out during actual living conditions are shown in Fig. 4. In most cases, the PCB concentration obtained during actual living conditions is less than results obtained by sampling according to ISO 16000-1.

The emission characteristic of steam volatile organic compounds like aldehydes is additionally influenced by the humidity of the indoor air. Increasing humidity increases the emission. Figure 5 shows the influence of room temperature, air humidity, and ventilation for formaldehyde in indoor air.

The Effect of Absorbing Effects (Sinks)

Due to specific physical characteristics of indoor air pollutants, longer-lasting indoor air contamination leads to relevant concentration on secondary areas in the room. One of the consequences is that these contaminated areas affect as a secondary source. Besides walls and floors, house dust is an important sink. Table 4 shows the

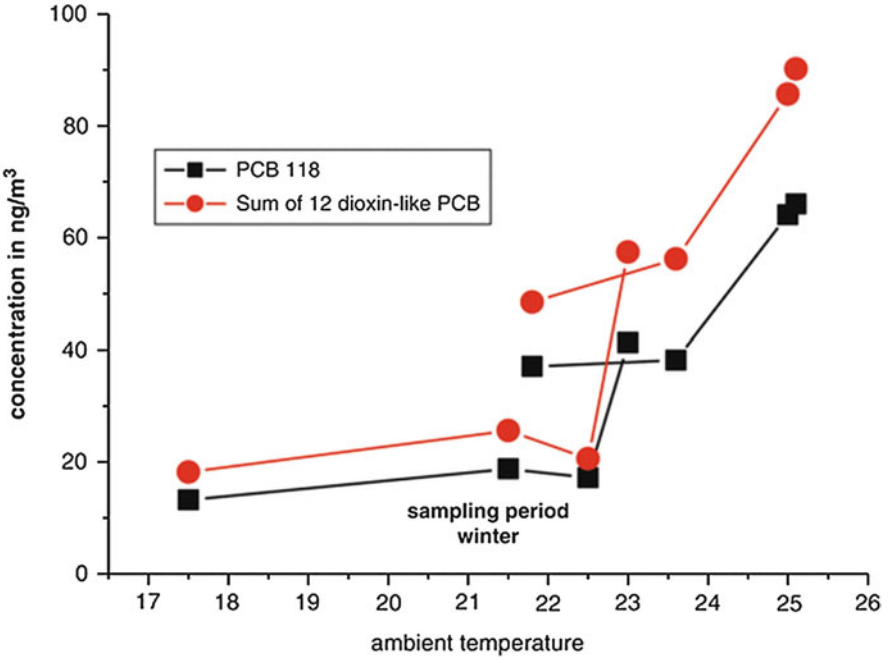

Fig. 3 Influence of room temperature and season on the concentration of PCB 118 and the sum of 12 dioxin-like PCB in indoor air (Volland et al. 2006)

contamination of house dust in buildings contaminated with softeners or flame retardants. Both sources, secondary contaminated building materials and contaminated house dust, may raise the indoor air concentration.

Conclusion

The given examples illustrate the influence of the indoor environment (temperature, humidity, season, ventilation) as well as the specific physical characteristics of indoor air pollutants on obtained measurement results. In the most cases, the methods to determine organic pollutants in indoor air deliver accurate and comparable results. For determination methods of VOC and SVOC in indoor air, if sampling is carried out under comparable conditions, the **usual accuracy is in the range of 10–30%**:

The complex correlation of the given structural situation in a building and the variety of the indoor climate normally overlaps the analytical errors of the determination step. The sampling strategy, the time of sampling as well as the parameters influencing the emission rate of the source are dominating the quality of results measuring the indoor air quality. Reported results without detailed information about the sampling condition cannot be assessed.

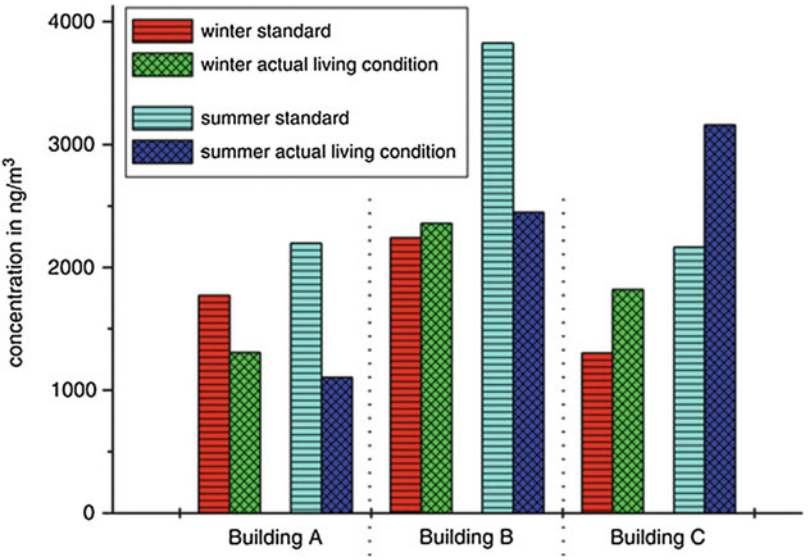

Fig. 4 Comparison of the concentration of PCB (sum of 6) in indoor air depending on sampling according to ISO 16000-1 (short time sampling) and sampling under actual living conditions, regarding the season, when sampling was carried out (Volland et al. 2006)

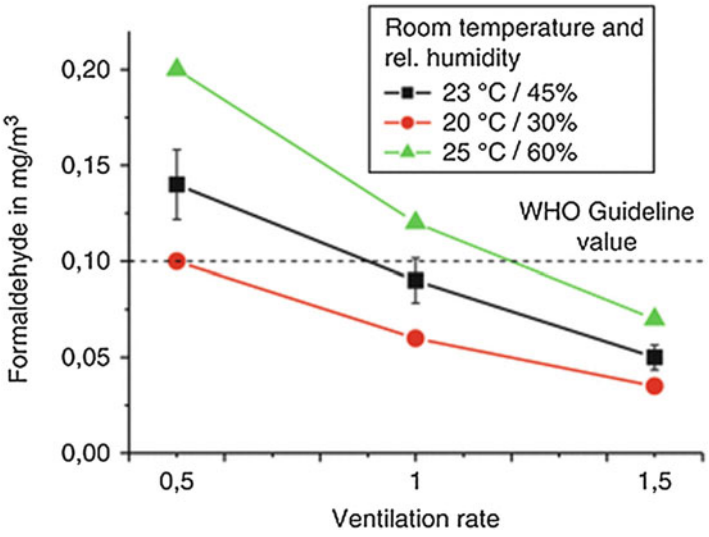

Fig. 5 Influence of room temperature, air humidity, and ventilation rate on the indoor air concentration of formaldehyde (calculated based on results reported by ISO 16000-3)

Higher differences between two measurement results are mostly **caused by differences of relevant parameters in the room during sampling**. These

differences in indoor environment can cause deviations of more than 100%. Thus, the assessment of results of the determination of VOC and SVOC in indoor air without the knowledge of the conditions during the sampling is fairly impossible. In the praxis often an exceeding of a guideline value is detected based on one single measurement. If the guideline value is based on a long-term average concentration, the **state of the art bans an assessment based on a single measurement**.

Cross-References

- ▶ Aims and Mission of Regulatory Toxicology
- ▶ Importance of Physicochemical and Physical Properties for Toxicological Risk Assessment
- ▶ Limit Values and Guideline Values in Regulatory Toxicology
- ▶ Principles of Analytical Chemistry for Toxicology
- ▶ Risk Communication: Challenges for Toxicologists and Other Risk Experts
- ▶ Working Areas of Regulatory Toxicology

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Benchmark Dose Approach in Regulatory Toxicology

26

Lutz Edler

Contents

Abstract

The Benchmark-Dose (BMD) approach aims at determining an exposure level/dose corresponding to a predefined change in response, the Benchmark Response (BMR), usually defined over background using all available dose–response (DR)

L. Edler (✉)
Dudenhofen, Germany

Formerly: Division of Biostatistics, German Cancer Research Center-DKFZ, Heidelberg, Germany

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information by fitting mathematical models to the dose–response data. The statistical confidence interval of the BMD (BMD-CI) accounts for the statistical uncertainty and the lower (one-sided) confidence limit, denoted BMDL, is used as reference point (RP) or point-of-departure (PoD) for the characterization of the risk of hazardous compounds replacing the no-observed-adverse-effect level (NOAEL) when sufficient DR data are available. Concept, scope of application, prerequisites for conduct, and key check points of the application of the BMD approach are presented and guidance is given for regulatory practice. The use of the BMD-CI for establishing a Health Based Guidance Value (HBGV) or a Margin of Exposure (MoE) is outlined.

Keywords

Dose–response model · Point-of-departure · PoD · Benchmark dose · BMD · Model averaging · Hazard characterization · Risk assessment

Introduction

The assessment of human health risk from the exposure to hazardous compounds has been omnipresent for scientists, regulators, and legislators in modern societies. Next to exposure assessment is dose–response assessment, a critical step for the risk characterization, and its outcome is a unanimously agreed base for decision making to protect human health. Measurement of doses and identification and description of adverse and toxic effects have challenged many scientific disciplines, in particular, toxicology, biology, and medicine. Ranging wide into the last century, the dominant focus was risk elimination and the definition of a "safe" dose below which no adverse/toxic health effect would exist. A long used concept has been the assumption of a threshold dose below which, theoretically or practically, no toxic effect would exist or might occur. From this concept evolved the NOAEL approach. Another early concept was low-dose extrapolation and the identification of doses at which none or no appreciable risk would exist.

Conceptual problems with the existence of such limits and practical limitations of measuring doses and responses initiated a more holistic view on dose–response data from toxicity studies in the second part of the last century, and methodological research on dose–response modeling became part of risk assessment, supported by the increasing availability of computational and statistical methods and data bases of toxicity data, for example, from the US National Toxicology Program (NTP). Initiatives of the US Environment Protection Agency (EPA), European Food Safety Authority (EFSA) and joint action of the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) through its Joint FAO/WHO Expert Committee on Food Additives (JECFA) have fostered comprehensive dose–response assessment for risk assessment for all relevant hazardous compounds. These international regulatory bodies and many national bodies apply Benchmark-Dose approach since about 2000. A search in PubMed in March 2021 resulted in

almost 1200 hits for "benchmark dose" and more than 900 hits for "benchmark dose" and almost 700 hits when restricting the search by adding "risk assessment." The BMD approach has meanwhile evolved to a state-of-the art for the extrapolation to low doses, the characterization of dose–response, the derivation of risk estimates, and the establishment of health-based guidance values (HBGV).

This development was supported strongly by methodological research in published literature, comprising mathematical modeling and statistical inference to identify reference doses that can be used for the risk characterization of hazardous compounds. Statistical software has been developed which could be applied by regulatory bodies, contract research companies, and individual researchers.

Although the BMD approach is based on a rather simple concept and proceeds in few well defined steps, as outlined below, all these steps allow for specific assumptions and modifications, often tailored for the risk assessment problem given by risk management and accounting for the type and amount of dose–response data available. Choices between options by groups of researches and expert groups led occasionally to diverting results and decisions for the same compound. This chapter informs on the relevant elements of the BMD approach and its application. A systematic outline of the approach and explanations of dose–response modeling methodology might sharpen the view on individual BMD calculations and help judging quality of the method and judgment of both validity and uncertainty. It should also be noted that the establishment of the BMD approach for regulatory toxicology has by now not reached its final status. Matter in fact, all three bodies mentioned above (FAO/WHO, EFSA, and US EPA) are currently, often jointly, engaged to improve and refine the use of the BMD approach. This chapter presents the current status and risk assessors should be alert for future news about the BMD.

Benchmark-Dose (BMD) Approach

The BMD approach has been introduced by Crump (1984) following discussions within US-NIH and US EPA when the paradigm of risk assessment was established by the US National Research Council (NRC 1983) as "A new method for determining Allowable Daily Intakes" with the explicit motivation for replacing the no-observed-adverse-effect-level (NOAEL) approach because of its potential shortcomings in risk assessment, see also Dourson et al. (1985) and Murrell et al. (1998). The shortcomings of the NOAEL, as well as the NOEL (no-observed-effect-level), have been reiterated and confirmed since then in numerous investigations and reviews, for example, U.S. EPA (1995, 2012), Edler et al. (2002), FAO/WHO (2009), and EFSA (2009). Methodological research on the BMD approach and initiatives for regulating human health and environmental risk assessment has boosted the use of the BMD approach during the past two decades when risk indices based on the BMD were used for food risk assessment, see, for example, the Food Safety in Europe (FOSIE) project (Barlow et al. 2002), the update of EFSA (EFSA et al. 2017b), guidance provided by U.S. EPA Davis et al. (2010), U.S.EPA (2021), and the update of

Chap. 5, "Dose-Response Assessment and Derivation of Health-Based Guidance Values" of FOA/WHO (2009).

The BMD approach was constructed to determine an exposure level or dose (the benchmark dose BMD) which produces a small but measurable adverse health effect in individuals of the exposed population. The allowed size of that effect is usually related to the background effect attributed to no exposure and it should be small enough to represent a relevant adverse effect and large enough for not critically depending on the mathematical models applied on selected dose–response data. To account for the statistical variation of the toxicity data, the statistical lower one-sided confidence bound of the BMD (the benchmark dose lower confidence limit BMDL) is calculated and used as Reference Point (RP) or Point-of-Departure (PoD) for the risk characterization (EFSA 2005).

The BMD approach controls the response level at the dose equal to the BMDL. This is in contrast to the NOAEL where the response level at the NOAEL is not controlled and can be rather high, in particular, when the sample size at the dose value equal to the NOAEL is small. The imprecision and the size of the response level at the NOAEL can therefore be large. A second advantage of the BMD approach over the NOAEL is that it attempts to use all available dose–response information by fitting mathematical models to all data. The NOAEL is somehow locked at one dose level of the toxicity study.

The BMD approach does not claim to be without uncertainty, but it makes the intrinsic uncertainty of empirical dose–response data more transparent. It investigates and describes the risk at or near a dose range where risk can be characterized and it allows extrapolation to dose ranges relevant for the assessment of human health risks, in particular for the range of human exposure. Therefore, the BMD approach is more transparent than previous approaches and requires a thorough analysis of available dose–response data.

The methods addressed in this chapter apply to the risk assessment of many types of contaminants, additives, etc., for various consumer products (food, drinking water, cosmetics, etc.) as well as for hazardous agents in the environment. However, the methods presented below differ from those applied for other risks such as radiation risk and from pharmaceutical medicines or risk of technical origin where the type of exposure is of different quality. A short overview on the BMD approach with a generic figure explaining the main features is given in ► Chap. 23, "Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds."

Dose–Response Assessment for Risk Characterization

In basic and applied research for risk assessment, toxicity studies are performed to identify sources of hazards and risks for human health and human environment. This research applies also for plant and animal health and the preservation of eco-systems. In the sequel of those studies, additional biological and toxicological investigations collect information to proceed from risk identification to risk characterization and further to risk management. Quantification of the risk is therefore essential and risk

indices such as the HBGV derived from dose–response data have evolved as gold standard for risk management. A HBGV provides quantitative information for risk managers and enables decision making to protect human health, for example, by defining a range of oral exposure that would be expected without appreciable health risk. This general approach applies to many types of human exposure and is applicable to many species and biological systems. It applies also to direct genotoxic and DNA-reactive carcinogens where not the HBGV but the margin of exposure (MoE) is the preferred risk index. Dose–response methods for the establishment of HBGV apply also to the MoE, see FAO/WHO (2009).

The link between hazard identification and establishment of HBGVs is the determination of a RP or PoD on a dose–response curve derived from toxicity experiments. The RP/PoD is a dose that corresponds to a specified adverse health effect, considered as response associated with a predefined risk. The preferred source of information for deriving an RP/PoD would be experiments or observational studies which have been prospectively designed for the specific dose–response assessment accounting for scenarios of the exposure of interest, possibly including the most vulnerable subpopulations. However, those studies are rare. On the other hand, many toxicity studies designed for the hazard identification provide sufficient data to quantify the risk and to determine a RP/PoD.

Note that in contrast to quantifying the risk for a given exposure, the determination of a RP/PoD is a sort of "inverse" estimation task: for a given risk level, say R^*, is an exposure level, say D^*, determined which assures with sufficient confidence that this risk level R^* is not surpassed when exposure does not exceed the level D^*. The establishment of a HBGV for risk management and regulation is based on that level D^* or levels derived therefrom. The HBGV is such anchored on the R* and therefore R* should have societal acceptance.

For an overview of approaches for the risk assessment of hazardous compounds see, e.g., Edler et al. (2002). The BMD approach differs from assessments which extrapolate from high to low doses (see also ▶ Chap. 23, "Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds").

Dose–Response Data Types

Dose–response (DR) data for hazard characterization originate almost exclusively from studies in humans or experimental animals. The focus here is on the assessment of risks of chemicals and compounds in food and diet. The relevance of food and diet as source of human exposure explains the abundance of quantitative risk assessment in that area. For the use of dose–response assessment for in vitro and genomic data, see e.g., Crump et al. (2010), Yang et al. (2007), and Long et al. (2018). The biggest challenge for the latter data sources is the construction of a robust qualitative relationship between the *in vitro* effects and the genomic alterations and the adversity on human health.

A clear concept of what is meant by dose and how dose is quantified is part of exposure assessment and crucial for the dose–response evaluation. Both, aspects of

toxicokinetic (TK)/toxicodynamic (TD) nature of the compound and statistical aspects of data should be considered (see also ▶ Chap. 28, "Toxicodynamic Models"). Regarding TK/TD and TD, one distinguishes in dose–response assessment between the *administered dose*, also called external dose and the *absorbed dose,* also called internal dose. The latter may be further specified as tissue concentration or concentration reaching the relevant target of adversity. Target doses can be obtained by case-specific biomonitoring. Most common candidates are concentrations of the toxic substance in blood or tissue or biochemical components (e.g., lymphocytes). The temporal nature of exposure is relevant when duration and frequency of exposure are factors to be considered. The field of food risk assessment distinguishes between acute and chronic exposure and separate HBGVs are established for the two types of exposure. Acute exposure can be quantified by the amount of toxins (e.g., mg toxin once given or mg toxic substance per kg body weight (BW) given once or several times within a short period of time, often 1 day only). The HBGV is then the acute reference dose (ARfD). In contrast, chronic exposure is usually quantified by the amount of toxin per kg BW and per day and hazard characterization assumes in general life-time exposure when establishing an acceptable daily intake (ADI) or a tolerable daily intake (TDI) as HBGV. Preferably, one would use individual body weight data for the analysis when the dose is in units of mg per kg BW per day. A special case of chronic exposure has been noted for toxic substances in drinking water where the dose is always presented as concentration, preferably as part per million or trillion (ppb or ppt). Concentration has been used as dose metric for animal health assessment in agriculture when the amount of toxin is related to the amount of daily feed consumption, for example, mg toxin/kg feed; see e.g., EFSA et al. (2017a) for the risk assessment of deoxynivalenol for humans and farm animals.

Transformation of doses into equivalent human doses (e.g., using allometric scaling) has been used in the past mainly when the NOAEL approach was applied or when the focus was on low-dose extrapolation. It has hardly been used with the BMD approach where the establishment of the link between the RP/PoD and the HBGV is constructed using uncertainty factors (FAO/WHO (2009)). Most toxicity experiments report the dose as measured in the study. However, the dose response data are often illustrated in graphics and, accounting for wide dose ranges, the original dose d is plotted often on log-scale with a graph of the response (on the vertical, say y-axis) versus $log(d)$ on the horizontal, say, x-axis. This occurs preferably when the doses were administered over several orders of magnitude (e.g., 0, 1, 50, 100, 500, 1000) or when the experiment was designed by purpose on logarithmic dose scale (e.g., 0, 1, 5, 25, 125, 625). It should be noted that the BMD approach has been developed for doses on the original scale and not the log scale and data analysis and graphical data presentation should always be separated. The fact that some model equations do have the term $log(d)$ in their definition formula should not be confused with the specification of dose at first place.

Essential for BMD modeling is the specification of the response related to observable toxicological effects identified as relevant for hazard characterization.

Whereas a toxic outcome informs usually on a wide spectrum of toxic endpoints, only those endpoints suit modeling that express adverse health effects or can be sufficiently associated with human health effects. Although a causal relation between an identified endpoint and exposure would increase its relevance for human hazard characterization, this relation should not be postulated as requirement since it might exclude informative relevant endpoints from a full risk characterization, besides conceptual and practical problems of the definition of causality. As a rule "only effects that link to adverse responses should be chosen for dose–response assessment" (FAO/WHO (2009)). Since the BMD approach is indispensably based on statistical methods, it is the statistical quality of the endpoint, in particular, its statistical scale that counts for DR modeling. This chapter will focus on the most prevalent categories of response data, the quantal and the continuous endpoints.

- **Quantal endpoints** (also denoted as dichotomous or binary) describe individual responses that inform exclusively on absence or presence of an effect, for example, assigning a 0 to absence and a 1 to presence. When considering several subjects exposed to the same dose, one summarizes the results of a group as the proportion of subjects with effect, the incidence of the effect. Is cancer the endpoint it is the cancer incidence.
- **Continuous endpoints** describe responses that are measured on a numerical scale, often as nonnegative values where 0 denotes absence of any measurable effect and where any positive value might occur under exposure. When considering several subjects exposed to the same dose, one may summarize again the response per group by the mean and the standard deviation (SD). It should be noted that from a statistical view the continuous endpoints can be subdivided further as of having an additive scale (i.e., differences of effects carry the relevant toxicological information, called interval scale) or a multiplicative scale (i.e., ratios of effects carry the relevant toxicological information, called relational scale). This distinction has possible consequences for the specification of the BMD model.

For other data types such as count data, ordinal categories of response, hierarchical nested endpoints (e.g., litter clusters) and multivariate endpoints see FAO/WHO (2009).

Except for data of human studies where a continuum of exposure doses could prevail, one usually faces a set of doses, $d_1, d_2, \ldots, d_I$ and a control group denoted by the "dose" d_0. These are ordered by their amount as $d_0 = 0 < d_1 < d_2 < \ldots < d_I$. The I + 1 groups are juxtaposed to the responses in the same order where, say n_i, subjects are exposed to dose d_i with n_i responses Y_{ij}, $j = 1, \ldots, n_i$, $i = 0,1, \ldots, I$. When considering tumor incidence in a carcinogenesis experiment the quantal response would be either $Y_{ij} = 1$ (for tumor) or $Y_{ij} = 0$ (for no tumor) in the jth animal of the ith group and the data are then summarized as $\{(d_i, n_i, p_i), i = 0,1, \ldots I\}$ with $p_i = r_i/n_i$ the proportion of tumor bearing individuals and if r_i denotes the number of tumors in group i. The continuous responses Y_{ij} represent usually measurements of a

biologically relevant effect, usually ranging between the value *0* and a maximum nonnegative value, say Y_{max} observed in the experiment. Such a maximum is difficult to fix because of heterogeneity between experiments.

Dose–Response Model (DRM)

Modeling DR data translates an anticipated relationship between the response *R* and the dose *D* into a mathematical and statistical framework to identify a DRM that describes the observed DR data and allows statistical inference and determination of risk indices such as a RP/PoD. The BMD approach is based on a two level concept where at first a mathematical function is defined that describes the expected functional dependence of response on dose. This mathematical function $\mu(dose)$ is an average or mean response function, a monotone increasing curve of *dose*. To account for the variation of response between the individuals of the population describes the second level the variation by a statistical distribution that may depend on dose. The distribution of the response, say P_R, is statistically defined by a density function $f(y)$ or a cumulative distribution function $Cf(y)$, y denoting the response. The $f(y)$ can be interpreted as probability of response equal to y and $Cf(y)$ as the probability that response is not larger than y. This statistical part of modeling is the stochastic element of a DRM and is the perquisite of any statistical inference. Both parts, the mathematical mean function $\mu(dose)$ and the statistical distribution P_R, constitute the DRM. If one knows the biological mechanism and the statistical variation one can put the two parts together in one model. In such an ideal situation one would just fit this model as the "true" model and estimate the true BMD statistically. The statistical fit to the observed data allows the calculation of the statistical confidence bounds such as the BMDL to be used as RP/PoD. However, this ideal situation is not the reality of risk assessment and both, the available knowledge about the dose–response relationship and in most cases, also about the response distribution is too limited to identify one single model and one single distribution as true. In contrast, the options for mathematical models and statistical distributions are literally infinite and therefore also for the BMD approach. DR modeling in risk assessment needs beyond mathematical and statistical means the input from biology and toxicology to accomplish model building. The more biological and toxicological information is available about the adverse health effects and their distribution the less uncertain is model building and the value of the RP/PoD. The sections below introduce sets of mean functions and distributions of response for building DRMs.

Before Starting the BMD Approach

Increasing practice with the BMD approach and discussions of diverging conclusions of regulatory agencies sharpened the awareness of risk assessors to control and to communicate their assumptions in BMD modeling. The most critical preparations are addressed in this section. Before starting a DR analysis of toxicity data, the risk

assessor is advised to identify the kinds of data available on dose and response, select the response and dose metric for the assessment, present and discuss the data of the study, for example, using graphical presentations, and the results of preliminary descriptive analyses, and judge the quality of data; all in front of the assessment. There have been ongoing discussions on the framework of the risk analysis within which DR modeling is embedded. At that interface between risk characterization and risk management, the latter might be consulted (Renwick et al. 2003; Abt et al. 2010).

Data Selection

Data selection for the BMD approach is an important first step of the BMD approach when the risk assessment problem has been formulated (Barlow et al. 2002; Renwick et al. 2003). Important issues include the human relevance of the data; however, also the quality of the original toxicity studies. General criteria for rating the quality of toxicity studies have been proposed by Klimisch et al. (1997) and were further specified by Schneider et al. (2009). When selecting DR data, one should consider, in particular, species, strain, and sex differences and their impact on the sensitivity of the test substance in target organs. Data requirements for mathematical modeling and quantitative methods have been formulated for eight approaches of food safety and food risk assessment by Edler et al. (2002) and for dose–response assessment and mathematical modeling in general by Barlow et al. 2002) including the BMD approach; for further notes on the selection of data see Edler et al. (2014):

- The observed shape of the DR curve alone is not a sufficient criterion for selecting DR data. The biological relevance of effects, for example, a deviation from monotonicity, must be weighed against the statistical significance of model fitting. Statistical model selection may not overrule available biological DR information.
- Attempts to qualify data through prescreening DR data for their suitability for the BMD approach may not be without arbitrariness due to the multiplicity of testing and the absence of statistical rules of how to set the significance level for a sequence of goodness-of-fit tests.
- Testing for the presence of a DR relationship, for example, using a trend test, could be misleading, when all effects remain below the primary chosen level of the BMR.
- An in-depth examination of the utility of studies is recommended before a DR analysis is performed.
- Most relevant for the size and precision of the BMD and BMDL are number and range of dose levels.

It was also emphasized that suitability of data for the BMD approach should not be separated from the quality of the toxicity study itself. Although agreed criteria to judge the quality of a study for a BMD analysis are difficult to establish and at

present not available, existing quality standards of regulatory toxicology regarding the test material used in the bioassays of the studies and guidance available for the conduct and reporting of bioassays (including, for example, genetic origin, housing, and health status of the animals) might be used for BMD analysis.

Statistical expertise should inform on the practicality of DRMs in view of the available data; this refers in particular to the design of the toxicity study and its influence on the modeling. Notice, however, that precise statistical methods for defining optimal designs for BMD modeling do hardly exist because of the statistical complexity of nonlinear regression. However, it has been noted that studies with more dose levels and less animals per dose would be preferred over studies with only few dose groups and a large number of animals in each (EFSA et al. 2017b). Deficiencies of a study design usually lower the BMDL, in striking contrast to the behavior of the NOAEL which tends to increase with inferior designs and gets such less protective. DR modeling using the BMD approach challenges the overall quality of the toxicity database. Some researchers suggested running the BMD analysis over all data sets of toxicity studies identified for risk assessment questions as a sort of screening for good quality DR data.

The above points referred primarily to data from animal experiments. Recommendations for the selection of human dose–response data are less developed; however, some guidance for the use of epidemiological data for dose–response assessment can be found in Herz-Piccioto (1995), Steenland and Deddens (2004) and van den Brandt et al. (2002).

Model Selection and Specification

A standard set of mathematical models of the mean response evolved for the use of the BMD approach in particular, for quantal and continuous data. Prominently to mention are the models of the software packages BMDS (U.S. EPA 2021) and PROAST (2020), see also Varewyck and Verbeke (2017). Table 1 lists by name single models for quantal and continuous data which have been repeatedly used so far (EFSA et al. 2017b; FAO/WHO (2009), Shao and Shapiro 2018). This lists single models (e.g., Logistic and LogLogistic model) and single models from model families (e.g., the Multistage family for quantal data and the Exponential and the Hill family for continuous data). Publications and guidance of different agencies vary in the reporting and handling of the models and model families. Although a tailored selection and definition of models can be a preferred option, there has been agreement among modelers to consider at least the models listed in Table 1 as default set for routine BMD analyses. The set of models has been proved flexible enough to cover a wide range of dose–response relationships. The newest version of U.S. EPA (2021) extended this list, in particular, for continuous data by variants of polynomial models, including the linear model, and a set of models evaluated by Bayesian models.

For the mathematical expression of the single models, see their presentation in the various guidance documents and related publications. The mean models are

Table 1 List of most used mathematical models of the mean response for quantal and continuous data

Quantal data	Continuous data
Probit	Linear
LogProbit	Power
Logistic	Exponential family
LogLogistic	M2
Weibull	M3
Gamma multi hit	M4
Multistage family[a]	M5
1-stage (QuantalLinear)	Michaelis-Menten
Multistage Cancer	General Hill
2-stage	Hill family
General Multistage	M2
Dichotomous Hill	M3
LVM[b] Exponential	M4
LVM[b] Hill	M5

[a]The multistage models is a general family of models from which only models assuming very few stages have been used for BMD
[b]LMV: Models used in PROAST (2020) which assume a continuous latent variable that describes the adverse health effect for a quantal data

given by mathematical equations for *μ(dose)* as function of the dose. The values of the model parameters are determined by fitting the model to the DR data. As important as the mathematical equation are the basic constraints of the model parameters. They locate and shape the function such that it fits the DR data in the observable ranges of the doses and the responses and they are an essential element of the model.

For illustration, see the mathematical expression of the Logistic and LogLogistic model for quantal data:

$$\text{Logistic}: \quad \mu(dose) = \frac{1}{1 + \exp(-a - b dose)}, \quad 0 \le dose < \infty \quad \text{with } -\infty < a < \infty \text{ and } b > 0 \tag{1}$$

$$\text{LogLogistic}: \mu(dose) = c + \frac{1 - c}{1 + \exp(-a - b \log(dose))}, 0 < dose < \infty \quad \text{with } -\infty < a < \infty, b > 0 \text{ and } 0 \le c < 1. \tag{2}$$

All models and members of model families are defined by their structural form and the model parameters with the basic constraints. This allows doses and responses to range over a large range of values limited only by monotonicity and avoidance of artifacts (such as negative incidences). The slope parameter, that is, the parameter b in Eqs. (1) and (2) above, is always positive for quantal data. For continuous data, primarily, two model families of the mean have dominated dose–response

assessment in regulatory toxicology: the Exponential and the Hill family. From a mathematical view, the mean models resemble those for quantal data. However, the response range is completely different. It ranges usually between 0 and any value with no fixed maximum. Depending on how effects and effect sizes are defined, a response can also be negative. As consequence, the definition of the adverse health effect and the specification of the BMR are for quantitative responses much more demanding. For convenience, a quantitative response is throughout this chapter represented by nonnegative values indicating an increase of severity with increasing values. The most general four-parameter exponential model is given here for illustration (Exponential M 5):

$$\mu(dose) = c + a \ \exp\left(-b\ dose^{k}\right) \text{ for } 0 \le dose < \infty \quad \text{with } b > 0, k > 0 \text{ and case dependent restrictions on } a \text{ and } c \tag{3}$$

The presentation of the mean models has been extremely inconsistent in published literature and, unfortunately, also in regulatory guidance. The formats presented in EFSA et al. (2017b), U.S.EPA (2021), and update of Chap, 5 in FAO/WHO (2009)) are not identical. The crucial point is, however, not the form of an equation in a guidance document. Decisive is only the way how the model and the distribution is implemented in the software and how the computational fitting algorithm is constructed. This requires statistical and computational expertise for understanding. Unfortunately, the code of software is rarely available and changes with versions of the software.

Note that the LogLogistic model for the mean response given by Eq. (2) cannot model incidences at the $dose = 0$ directly: $log(dose) = log(0)$ is no real number; mathematically $log(0) = -\infty$, minus infinity. Therefore, when this model and any other model with $log(dose)$ is used the software implementation uses instead of the dose $d = 0$ a very small positive number, for example, $0.00001 = 10^{-5}$ to avoid minus infinity. In other words, the dose-interval $0 < dose < \infty$ is replaced by $10^{-5} < dose < \infty$.

For models for continuous data is the slope parameter positive. However, the curve can turn upwards or downwards depending on direction of the effects of the toxic substance. As noted above this chapter considers increasing severity. Basic constraints of the parameter a and c serve for that. Each single model for the mean response of the suite of models displayed in Table 1 is defined by the values of 3–4 parameters. Parameter k is often denoted as potency in case of quantal and shape in case of continuous data. For further interpretations of parameters and their combination, see, for example, EFSA et al. (2017b).

By imposing additional constraints on model parameters, one can restrict the possible range of modeling. This is still an option in the BMDS software of U.S. EPA (2021) and has to be used with caution since it restricts modeling such that results of the same models applied to the same data produce different BMDs and/or BMD-CIs and therefore different RP/PoDs. Differences of established HBGVs up to an order of magnitude could be traced back to the use of additional constraints during modeling. An often used constraint excludes dose–response curves which

have a steep (up to infinite slope) at the origin (i.e., at $dose = 0$). Those curves can be excluded by restricting the slope at $d = 0$ to be not larger than a fixed value, say 1, U.S.EPA (2012). However, it has also been argued that this option should be avoided and that the full range of model parameters should be allowed for each model (EFSA et al. 2017b). It has also been recommended to examine visually the shape of the fitted DR curve and check how the values of the parameters in a model and the BMD and the BMDL values react on constraints made during model fit. As a default, it was recommended not to constrain the model parameters as long as there are no convincing biological arguments (EFSA et al. 2017b). From a statistical point of view, keeping the space of the model parameters as wide as possible is fortunate, since it reduces the chance that model parameters hit boundaries in the parameter space with all subsequent computational difficulties. Despite intense discussions at various rounds of harmonization of dose–response modeling for hazardous chemical compounds, there is still no agreement on the use constraints to guide risk assessors in the use of constraints when fitting single models. It was only recently, when model averaging (MA) and Bayesian approaches became available for the BMD approach, that the concerns on the use of constraints diminished when those statistically more complex methods replaced single model fitting.

The update of FAO/WHO (2009) attempts also to generalize model building for mean response by defining a hyper-family of models that includes almost all single models of Table 1 by requesting for continuous data the format

$$\mu(dose) = \quad c + a\,F\left(\,dose^{k}, b\right) \qquad \text{for } 0 \le dose < \infty \tag{4}$$

with the four parameters *a,b,c,* and *k* and a general function *F* where the effect of the dose is strictly modeled as a power of the dose with exponent $k > 0$, mainly responsible for the shape of the mean response curve. This general format holds also for quantal data given a minor modification: the set of parameters is reduced to *a, b,* and *k* when setting $c = 1-a$. In strict mathematical sense, it does, however, not cover the quantal models that depend on *log(dose)*. Therefore, for quantal data one should use two formats:

$$\begin{aligned} \mu(dose) = \quad & a + (1-a)\,F\left(dose^{k}, b\right) \qquad \text{for } 0 \le dose < \infty. \\ \mu(dose) = \quad & a + (1-a)\,F\Big(\,(\log(dose))^{k}, b\Big) \qquad \text{for } 0 < dose < \infty. \end{aligned} \tag{5}$$

In order to select a DRM, one has also to specify the distributional part of the model as the second component of the model building of the BMD approach. It defines the statistical distribution of the response, P_R. For individual quantal response, the distribution, depending on $dose = d$, is the Bernoulli distribution. The probability of a response is *p(d)* and for nonresponse *1-p(d)*. The number of responders in a group of *n* individuals has then a Binomial distribution with two parameters *(n, p(d))* and the (theoretical) mean is *np(d)*. This term is modeled by the mathematical mean function. The BMD approach can therefore be restricted to the

summary data, that is, to the incidences, of the groups of the experiment using the data triplets (d_i, n_i, $p(d_i)$), for $i = 0,1, \ldots, I$.

For human data where each individual is exposed to a unique dose this distributional assumption works only when the data can be aggregated into a set of I categories where $I = 0$ would represent the background. Otherwise, when modeling individual data, there exists usually additional information for humans in the form of covariates describing relevant characteristics of each individual. This calls for more sophisticated epidemiological model building, see below.

The BMD approach used historically for individual continuous response the Gaussian Normal distribution $N(\mu(d), \sigma(d))$ as the default with mean $\mu(d)$, depending on dose d and a standard deviation (SD) $\sigma(d)$ that may depend on d. Were the DR data available in I dose groups and a control $(I = 0)$ with n_i subjects exposed to dose d_i and the responses y_{ij}, $j = 1, \ldots, n_i$, $i = 0,1, \ldots, I$, the individual responses are modeled as

$$y_{ij} = \mu(d_i) + e_i \qquad \text{for each group } i = 0, 1, \ldots, I \tag{6}$$

where e_i is an additive statistical error term describing the statistical variation of the responses at dose d_i. The observed means are denoted as m_i and their observed standard deviations as s_i^2. The quadruplets (d_i, n_i, m_i, s_i^2) summarize then data and are the input for the software.

The assumption of the Gaussian Normal distribution has two handicaps when being applied to biological data. One stems from its mathematical property to be defined on a response range $-\infty <$ response $<\infty$, the other is due to its symmetric distributional shape around the mean. Both properties do not fit toxicological responses when they are nonnegative or positive and when they exhibit a skewed distribution with a large portion of low responses sticking together and a relative small portion of the large responses reaching far out to high values. Very high responses were sometimes denoted as "outlier" and were discarded from the dose–response analysis. A deletion of data is, however, not recommend since even the Gaussian Normal distribution would allow for high values and it is hard to decide whether a high value still belongs to a single statistical distribution or to a component of a mixture of several distributions. Whereas one may neglect the first handicap of the response range $-\infty <$ response $<\infty$, by assuming that the Gaussian Normal may still serve as a good approximation, when the observed means are all positive, skewness causes a problem and needs to be accounted in modeling, in particular, when it is biologically plausible. The log-normal distribution was therefore introduced rather early (Slob 2002) for the BMD approach as a second default distribution for continuous data. It is a distribution with nonnegative values skewed to the right, allowing relative high values without the suspicion that they are erroneous measurement or belong not to the assumed distribution. The lognormal distribution is for sure not the only statistical option for nonnegative right skewed data; however, it is a simple one and mathematically easy to handle because of its direct relationship with the Gaussian Normal:

The distribution of the response Y is lognormal if the logarithm of Y is Gaussian Normal. (7)

In other words, would we use instead of the response *Y* as observed the logarithm *log(Y)*, we could analyze these transformed responses *log(Y)* with the methods we do have for the Gaussian Normally distributed data. This has been often used as an option in statistical inference when the consequence of the statistical computation was qualitative, for example, in statistical testing using the analyses of variance (ANOVA) or other regression methods. Unfortunately, this does not apply for the quantitative analysis of the BMD approach where the size of the response or its changes, the BMR, is the quantity of interest; see U.S.EPA (2021) for concerns of using *log(Y)* data. When assuming the lognormal distribution, one should be aware of the properties of this distribution and the specific forms of their mean and SD. It follows from Eq (7), then

$$\text{mean of lognormal} = \exp\left(\mu + 0.5\sigma^2\right) \tag{8}$$

$$\text{SD of lognormal\_} = \exp(\mu)\,(w(w-1))^{0.5} \text{with } w = \exp\left(\sigma^2\right). \tag{9}$$

Accounting for the more complex form of the mean of the log-normal distribution, the BMD modeling uses in place of the mean of the distribution as the center (average) of the statistical distribution the median:

$$\text{median of lognormal} = \exp(\mu) \tag{10}$$

which depends on μ only. This is concordant with Eq. (7) since *log (exp(μ))* = μ. The median of the lognormal is identical to geometric mean and reflects such the multiplicative background of the lognormal distribution (Slob 2017). The geometric SD of the log normal distribution is equal to *exp(σ)*. These mathematical relationships have been obscured when using the terms normal scale and lognormal scale without explicit mathematical detail.

Another method of the BMD approach used for continuous data has been the so-called hybrid approach where the originally available continuous response data are transformed into quantal responses by introducing a critical cut-off for the effect, see Crump (1995), Gaylor and Slikker (1990), Falk Filipsson et al. (2003), and U.S. EPA (2021).

Specification of the Benchmark Response (BMR)

The specification of the BMR constitutes the most essential step in the BMD approach since it quantifies the size of an effect for response evaluation, at which a RP or PoD is anchored in the dose–response relationship. The value of the BMR links the DR modeling quantitatively with the adverse health effects for quantitative hazard characterization. In contrast to other means of hazard characterization, for

example, the NOAEL approach or other semi-quantitative approaches, the BMD approach is fully quantitative and specific for an effect size.

Therefore, before analyzing, DR data type and the size of BMR must be specified. In the short history of the BMD approach has this been the most important but also most controversial decision to be made. It links toxicological and statistical reasoning and needs the cooperation of both disciplines. Toxicological expert knowledge should drive the choice of the critical endpoint and should elaborate possible quantitative sizes of changes over background that account for biological and optionally also medical relevance and for public health consequences. Statistical expert knowledge is challenged to translate toxicological facts into statistical entities and response metrics to be used in the BMD modeling accounting for the statistical quality of the data. It has been advocated that the BMR should be set equal to a low but measurable response level reflecting an effect that is negligible or nonadverse. Obviously, a too low BMR would normally result in an extrapolation outside the range of the observed data and induce severe model dependence of the RP/Pod. Therefore, a practically useful BMR cannot be set too low and it may in some real life dose–response assessment be associated with substantial risk. The BMR specification could therefore only be feasible as a compromise between the ideal property of reflecting nonadverse or negligible effects on the one side and the practical needs of statistical valid estimation of the BMD and the BMD-CI that avoids bias.

For quantal response data is the critical endpoint the incidence expressed as proportion or probability. This probability of the occurrence of the event is denoted *R(d),* indicating its dependence on dose *d*. The response is defined by an expression that compares *R(d)* with the risk at background *R(0)* on an additive or multiplicative scale:

$$\text{added risk } R(d) - R(0) \tag{11}$$

$$\text{extra risk } [R(d) - R(0)]/[1 - R(0)] \tag{12}$$

The extra risk is standardized by the maximum possible risk added to background *1−R(0)*. Note that for quantal data the mean function $\mu(d)$ is trivially related with the risk *R(d)* via $R(d) = p(d) = \mu(d)/n$. Extra risk has been widely used as default metric of response for quantal data and is given on a percentage scale, theoretically, ranging between 0% and 100%. A BMR for extra risk $p,\ 0\% < p < 100\%$ defines a BMD_p as

$$\left[R\left(BMD_p\right) - R(0)\right]/[1 - R(0)] = p = BMR_p \tag{13}$$

indicating the chosen BMR level p as subscript, usually also expressed on the % scale. For example, the BMR =10% is denoted BMR_{10} and its BMD and BMDL as BMD_{10} and $BMDL_{10}$, respectively. The BMR of 10% has been the default critical effect size for tumor incidence over background. It is the dose where the incidence is not larger than 10% and the $BMDL_{10}$ is therefore the dose where the incidence is (statistically) unlikely larger than 10%. In precise statistical wording, the $BMDL_{10}$ is the lower (one-sided) statistical 95% confidence level of the BMD_{10} and the (extra)

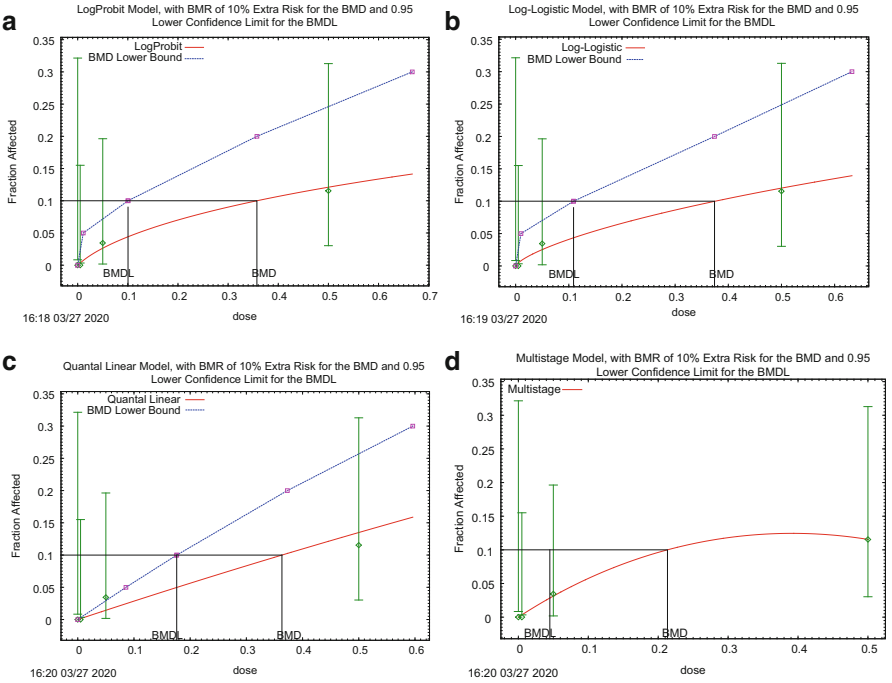

Fig. 1 The Log-probit (**a**), LogLogistic (**b**), QuantalLinear (**c**), and Multistage-2 (**d**) model fitted to the incidence of haemangiosarcomas in rats after exposure to sterigmatocystin reported by Maekawa et al. (1979). Each graph shows the incidences with 95% confidence intervals, the fitted model curve (red) and above that the (pointwise) 95% confidence bound of the fitted model used for calculation of the BMDL using U.S. EPA BMDS software

risk at a dose equal to $BMDL_{10}$ is with a probability of 5% not larger than 10%. It should be noted that the BMR (additive or multiplicative) is not defined as a change of the *observed risk* (observed mean response μ), but of the *predicted risk*, predicted by fitting the model $\mu(d)$ to the data. This distinction is important since the fitted model does not hit the observed background response (the observed incidence of the control group) exactly. Simply adding the chosen BMR value to the observed background response on y-axis of a BMD plot is therefore not identical with the intersection of the fitted curve with the y-axis at dose = 0, see Fig. 1 in EFSA et al. (2017b).

The default choice of 10% as BMR for an extra risk BMR has been criticized as of being too large as an acceptable human risk level for carcinogenic compounds. Indeed is a BMR of 10% too large for the assessment of human cancer risk. Acceptable risk levels ranged between 10^{-4} and 10^{-6} corresponding to BMRs of 0.01% and 0.0001%, respectively. However, setting the BMR much lower than 10% does not account for the sensitivity of most cancer bioassays, and therefore, the default value of BMR = 10% for quantal data must be viewed as a compromise between measurability and relevance of effects.

The specification of a BMR for continuous data is conceptually similar, but more difficult since the scale of the endpoint is no more bounded and the nature of the continuous effects is much. On the other hand, BMRs defined for continuous data do have the potential to better reflect effects that are negligible or nonadverse. There exist again the two options of an additive and a multiplicative metric for the effect size in comparison with the background effect *Y(0)*. With *Y* denoting the critical endpoint for the BMD modeling, one defines

$$\text{added effect } Y(d) - Y(0). \tag{14}$$

and

$$\text{relative effect } [Y(d) - Y(0)]/[Y(0)] \tag{15}$$

where BMR is again a portion $p > 0\%$. For the multiplicative scale, standardization is versus background effect *Y(0)* only, since a maximum effect is difficult to precise for quantitative effects.

A third definition of the BMR relates the added effect size *Y(d) – Y(0)* to the variation of the data observed for the controls, that is, the standard deviation of Y(0), say SD-control (variation standardized effect):

$$[Y(d) - Y(0)]/\mathrm{SD}_{\text{control.}} \tag{16}$$

The choice between an additive and a relative metric for the effect size should be made on a thorough examination of the scale of the continuous endpoint. The relative effect has been the most chosen BMR definition in practice using for the mean function $\mu(d)$ a relation between BMD and BMR that is similar as for quantal data. In contrast to quantal data, there is no more an upper bound for p and it is prudent to choose small percentages for the relative effect with p as subscript when defining the BMD_p by.

$$\left[\mu\left(BMD_p\right) - \mu(0)\right]/\mu(0) = p = BMR_p \tag{17}$$

For example, a BMR =5% is denoted BMR_{05} and has been a default critical relative effect size over background in many BMD approaches for continuous data resulting in BMD_{05} and BMDL_{05} values. The BMD_{05} is then the dose where the relative effect is not larger than 5% and the BMDL_{05} is a dose where the relative effect is (statistically) unlikely larger than 5%. Recent guidance has criticized this default and requested justification of any BMR value chosen (EFSA et al. 2017a).

When using the variation standardized effect as metric the BMD_p is defined by

$$\left[\mu\left(BMD_p\right) - \mu(0)\right]/\mathrm{SD}_{\text{control}} = p = BMR_p \tag{18}$$

This metric can be generalized by replacing the calculated standard deviation of the control in the denominator by other variation estimates, for example, 2SD

instead of 1SD. The 1SD has been the preferred use of US-EPA and is a default in BMDS software (U.S.EPA 2021). The standardization by the SD was justified by the argument that it allows a better comparison of BMDLs from different experiments. However, the dependency of the RP/POD on the variation of single experiments has also been considered as a weakness since the RP/PoD values would then depend not only on the quality of the data but also on all other study-specific factors that may increase or decrease that variation. It was also noted that the extrapolation of RP/PoD for humans in the risk characterization step would be more dependent on the animal data than when using the relative effect metric (EFSA et al. 2017b).

It should be noted that US-EPA guidance for the BMDS software uses instead of the term BMR the term "Benchmark Response Factor" (BMRF) with the defining eq.

$$\mu\left(BMD_p\right) = \mu(0) + BMRF_p\mu(0) \tag{19}$$

This presentation may have advantages for understanding the software.

The update of FAO/WHO (2009) integrates the specification of the BMR for continuous data into a tiered approach with "consideration of biological and statistical significance, human relevance and the existence or absence of a dose–response relationship":

1. At first, one should clarify whether a biologically relevant adverse effect size can be defined for the critical endpoint. Therefore, historical data may be used. If a cut-point can be defined, such as in the hybrid approach, a BMR similar as for a quantal response data may be used.
2. If tier 1 does not work, an expert decision can be made "involving risk assessor (s), statistician(s,) toxicologist(s) and/or clinician(s) who all collaborate to define the quantitative definition of adversity and consider type and severity of the effect, the background variability and the mode of action leading to the effect." One might use a range of BMR values in place of a fixed numerical value.
3. If tiers 1 and 2 would not lead to a definite BMR level, the BMR approach itself might not be an option at all and dose–response assessment might proceed with modeling the data to extract dose–response information such as "investigating fold changes, looking at differences from background estimating response from the DR curve" to obtain eventually a range of quantitative values useful to derive some reference values that characterize the hazard, for an ad hoc judgment or a MoE type assessment. It was stressed that those values should not be used to establish a HBGV.

There have been tendencies, in particular for continuous data to use BMRs larger than the 5% or 10%, for example, response levels of 25% and even 50%. The use of such more "central" indicators of dose response may be indicated for the comparison of the toxic potency of compounds but hardly for risk characterization and they are not recommended for the establishment of HBGVs and MoEs.

Model Fitting and Model Acceptance

In principle, model fitting is a method to find those values of the model parameters that result in a DR curve which is over the whole observed dose range as close as possible near the observed data points. This is easy when fitting a straight line to data where the two parameters, background (value at dose = 0) and the slope of the curve are the only model parameters. They are estimated best in linear regression with an explicit mathematical function. This is directly related to the Gaussian least squares method. Note, however, that the optimality of straight line as the best fit of the linear regression and the interpretation of the estimates depends on the Gaussian normality assumption for the statistical distribution of the response. The complexity of curve fitting increases with the number of parameters, the nonlinearity of the model, and the choice of the response distribution.

If the BMR and the dose–response model (or a suite of models) have been specified, fitting model(s) uses established statistical and computational mathematical methods. Three model fitting options are currently available for the BMD approach, two frequentist approaches and the Bayesian approach including model averaging (MA):

(a) The frequentist approach results in a fully specified model often denoted as the "best fitting model" from which the BMD can be estimated and the BMD-CI calculated. The fitting can be described as statistical search method for parameter values which lead to a dose–response curve that fits best the observed data points.
(b) The modified frequentist approach identifies a small set of fully specified models by searching for those parameter values which lead to a dose–response curve that fits the observed data points reasonably good. Models of reasonable fit are identified by prespecified acceptance criteria. In a second step, the BMDs and BMD-CIs of those "acceptable models" are combined to derive a RP/PoD.
(c) The Bayesian approach is a statistical demanding machinery which determines a RP/PoD using all the selected models. This statistical methodology makes use of prior information on unknown elements in modeling using biological and toxicological reasoning. A special Bayesian approach is the so-called Bayesian MA which uses a weighing of models and allows an interpretation somehow similar to the modified frequentist approach.

The modified frequentist approach accounts for the practical experience of hazard characterization that the BMD approach should not aim to find the single statistically best fitting models but rather to identify all plausible models and their BMD/BMDL values that are compatible with the observed data and constitute a set of "acceptable" RP/PoDs.

Different from the frequentist approach, inform the methods of MA and the full Bayesian approach on the model uncertainty of the BMD estimates and the derived RP/PoD. For further details of all three fitting approaches, see EFSA et al. (2017b). For computational details, contact U.S.EPA (2021).

All model fitting approaches use statistical acceptance criteria which allow assessing the quality of the fit; in common language: How good do the chosen models fit the observed data? Software implemented for the BMD approach uses its way to find solutions for the estimation of the model parameters, the derivation of a BMD confidence interval, and the presentations of results, including a graphical illustration of what is called the best fitting curve. Details of occasionally complex fitting using mathematical modeling and computationally sophisticated computational procedures are often hidden in BMD analysis software. Recommended BMD software informs on the used statistical criteria for fit and reports key indices of the "goodness-of-fit" in its output. Users should be familiar with basic model acceptance criteria to understand and interpret uncertainty and validity of the BMD estimate, the BMD-CI, and the derived RP/PoD. It is important to get familiar with those criteria before the computational execution of BMD analysis such that appropriate software options can be set and the key quality indices are calculated. The concept of those criteria is shortly described next.

Visual inspection can be used to compare the quality of the fit of the final "best fitting curve" (or any equivalent graph) with the observed dose–response data in a graphic. This is a highly subjective procedure which depends on the experience and attitude of the risk assessor and their "angle of view" but also on the presentation of the final model and the data through the software. It is the distance in the vertical direction, that is, parallel to the y-axis in a coordinate plot which informs on the quality of a fit. It would be misleading to judge a perpendicular or horizontal distance. The maximum likelihood method reduces to a least squares fit when assuming a Gaussian Normal distribution where the squares of the difference between fitted and observed values (the residuals) count; related to the fact that for this distribution the maximum likelihood method reduces to a least square fit. The visual assessment is more complicated when assuming a lognormal distribution for the response. Visual inspection may fail when curve and data are transformed either by purpose to ease the construction of the methodology and software or by intention to ease presentation. Log-transformations of dose and/or response can distort to visual impression seriously.

The statistical standard for acceptance of a fit based on the (maximum) *likelihood approach* for the BMD analysis is the likelihood of the parameter estimates. Likelihoods are given in the scale of probabilities and have values between 0 and 1, mostly reported as logarithms $L = log(likelihood)$, the log-likelihood value of the fit. For a more detailed discussion and numerical examples of the log-likelihood values and their role for comparing models, see the first version of the EFSA Opinion (EFSA 2009).

BMD software reports L values for each model, often together with a CHI-square statistic value as an another index of "goodness-of-fit." Statistical P-values are reported for a likelihood ratio test and the chi-square test, by testing the null-hypothesis that the model fits the data versus the alternative that it does not fit. This P-value controls the error probability of rejecting the fit, given the fit was correct (type 1 error) and not the probability of main interest in fitting models, namely, the error probability of accepting the fit, given it was incorrect, that is, of no good fit (type 2 error). Therefore, the P-value of the "goodness-of-fit" testing can only be used as an indicator for the goodness-of-fit. A high P-value indicates good fit and a

small one a bad fit. Note, however, that the numerical size of this P-value depends on sample size and number of parameters estimated and in the case of grouped data on the number of experimental groups. A level of $P > 0.1$ (10%) or $P > 0.2$ (20%) has been used in model fitting to accept a model. Smaller levels indicate inferior fits and very small ones, say $P < 0.01$, indicate an unacceptable fit.

When models are nested, for example, when using the Exponential and the Hill model family for the continuous or the multistage model family for quantal data, the log-likelihood criterion can be used to find the "best" model within the model family.

The limitations of log-likelihood initiated about 50 years ago the construction of the *Akaike Information Criterion (AIC)* which accounts for the number of parameters, usually denoted as p, estimated during model fitting. The AIC is defined as $AIC = (-2\,L) + 2p$. It is a positive value indicating a good fit when being small and a bad one when being large, however, only as long as the calculation is in the same modeling context: The numerical value of the AIC cannot be used to assess an overall or absolute quality of a fit. The AIC corrects somehow for parsimony of model section when it increases its value with the second term $2p$ and punishes overfitting when using models with too many parameters. The AIC has been used in the revised version of EFSA (EFSA et al. 2017b) to build a flow-chart for the modified frequentist approach.

BMD Modeling in Practice

Dose–response assessment with BMD approach is determined by the availability of informative toxicity studies for hazard characterization. The quality of the data and the endpoints of adverse health effects determine depth and extent of BMD modeling. A prospective evaluation plan should be set up involving all parties which are interested in the outcome of the assessment (bodies of the regulatory agency, modelers and risk assessors, risk managers, and if available, risk communicators). This would help straight modeling and increase reproducibility of the approach and transparency of the assessment. First of all, a well-designed strategy for handling the data and the potential critical effects of the selected studies is needed.

One Data Set

It is not unusual in risk assessment that hazard identification supports the evaluation of one critical endpoint of one data set only. An example is cancer incidence when one set of pivotal dose–response data has been identified as most relevant and sufficiently informative for example, a NTP study performed according to an established design and published transparently with public access to all relevant data. BMD modeling for one data set and one critical endpoint is illustrated next for the incidence of hemangiosarcomas in rats as critical endpoint in a data set identified as pivotal for the risk assessment of the mycotoxin sterigmatocystin EFSA (2013). The data were obtained from Maekawa et al. (1979) who exposed rats to

sterigmatocystin (STX) at doses 0, 0.005, 0.05, and 0.5 mg STX/kg b.w. per day and observed hemangiosarcomas at the incidences of 0/11, 0/27, 1/29, and 3/26, respectively.

The incidence in the three dose groups was very low and indicated an increasing trend with dose. The Cochran-Armitage test for a linear trend showed with a P-value of 0.23 of no statistical significance. This should, however, not prohibit a detailed dose–response analysis using models for quantal response as shown in Table 1. This subsection illustrates the BMD approach in practice by discussing the basic steps of the BMD modeling when fitting of a single model and then a suite of models.

Since the doses were logarithmically spaced between 0.005 and 0.5 mg/kg BW per day, the fit of the LogProbit model as simple model with a mean function depending on log(dose) appears reasonable. Using BMDS software (Version 2.60), the BMD_{10} was estimated as 0.36 mg/kg BWper day for the BMR_{10}, corresponding to 10% cancer incidence, noting that the highest observed incidence was 12% at the highest dose. The lower one-sided 95% confidence bound $BMDL_{10}$ was calculated as 0.10 mg/kg BWper day ranging between the second and third dose. Figure 1a shows the fitted curve of the LogProbit model and Table 2a the details of the fit, with BMD_{10} and $BMDL_{10}$ at the right together with the log-likelihood value of -13.7786 and the AIC $= (-2 \text{ loglik}) + 2\text{ p} = 31.5573$. The number of model parameters was $\text{p} = 2$. Both the GoF test and the CHI-square test indicated with their rather large P-value of 0.88 and 0.92, respectively, a good fit, visualized in Fig. 1a. The difference between the observed incidences of 0, 0, 1, and 3 in the four groups and the estimated incidences of 0, 0.093, 0.765, and 3.148, respectively, of the fitted curve (the so-called expected incidence) was small supporting the quality of the fit.

Acceptability of a model can also be assessed by comparing the fit of a selected model with the fit of the so-called *FULL model*. The full model simply consists of the observed (mean) responses at each applied dose and it is point-wise mean function where the fitted curve takes the value of the observed response at the respective dose. It "interpolates" the observed DR curve and the number of parameters equals the number of groups. If a model's fit is not significantly worse than that of the *FULL model*, for example, when $P > 0.05$ one may accept the specific model. In addition, an acceptable model should also explain substantial dose–response information by being statistically significant ($P < 0.05$) different from the *NULL model*. The *NULL model* stands for the nonexistence of a DR relationship, that is, the mean responses of control and dose groups are all on a straight line parallel to the dose axis. In summary, the statistical fit of an acceptable model should be statistically significantly better than the *NULL model* ($P < 0.05$) and not significantly worse than the *FULL model* ($P > 0.05$). In cases where none of the models pass the tests, visual inspection of the data may show that some models still adequately describe the observed DR. In that case, the decision to accept a particular model needs to acknowledge the high level of uncertainty of BMD estimate and the BMDL value. Note also that the choice of the significance levels is not stringent and nothing more than statistical defaults.

Table 1a informs on the fit of the *NULL* and the *FULL model*. The *NULL model* has the log-likelihood value of -16.4979 and the AIC $= 34.9958$ and the goodness-of-fit is rather weak with a $\text{P} = 0.13$, in concordance with the outcome of the trend

Table 2 BMD approach detailed by the dose–response assessment of the incidence of haemangiosarcomas observed in rats after exposure to sterigmatocystin reported by Maekawa et al. (1979). BMDs and BMDLs were determined for a BMR of 10 using US-EPA BMDS software

A: Detailed results of the fit of the LogProbit model using U.S. EPA BMDS software for a BMR_{10} (BMR=10% extra risk)								
Parameter estimates with 95% confidence interval								
Background incidence:	−0.94	−1.73 to −0.15						
Slope	0.33	−0.044 to 0.71						
Observed and expected incidence and their deviation expressed as scaled residuals								
Observed	Expected	Deviation						
0	0	0						
0	0.093	−0.305						
1	0.765	0.272						
3	3.148	−0.089						
BMD modeling characteristics of the fit of the LogProbit model								
Model	Number parameters	loglik	GoF test P-value	DoF	CHI-square test P-value			
NULL	1	−16.4979	0.13	3				
FULL	4	−13.6482	na	na	na			
LogProbit	2	−13.7786	0.88	2	0.92			
BMD result of the LogProbit model in mg/kg BW per day								
BMD_{10}	$BMDL_{10}$							
0.36	0.10							

B: Result of the fit of the suite of models using available in U.S. EPA BMDS software for a BMR_{10} (BMR=10% extra risk)								
Model	Param	loglik	GoF		CHI-Square AIC		BMD_{10}	$BMDL_{10}$
NULL	1	−16.4979	0.13	3	na	34.9958	na	na
FULL	4	−13.6482	na	na	35.2964	na	na	
Probit	2	−14.3615	0.49	2	0.57	32.7229	0.48	0.33
Logistic	2	−14.3789	0.48	2	0.57	32.7596	0.49	0.35
LogProbit	2	−13.7786	0.88	2	0.92	31.5573	0.36	0.10
LogLogistic	2	−13.8285	0.84	2	0.89	31.6569	0.37	0.11
Weibull	2	−13.8341	0.83	2	0.88	31.6682	0.38	0.11
Gamma	2	−13.8378	0.83	2	0.88	31.6757	0.38	0.12
M-QuantalLinear	1	−14.0289	0.86	3	0.81	30.0579	0.36	0.18
M-Cancer	1	−14.0289	0.86	3	0.81	30.0579	0.36	0.18−1.76[a]
Multitstage-2	2	−13.744	0.91	2	0.95	31.4880	0.21	0.045

bw body weight; na not applicable; GoF goodness-of-fit; DoF degree-of-freedom; loglik loglikelihood; AIC Akaike Information Criterion

[a]only model for which BMDS provides also the upper bound of the BMD-CI

test. The *FULL model* as model with "perfect" fit of the observation has likelihood value of −13.6482 and the AIC = 35.2964 to be compared with those of the fitted model. Compared with the *NULL*, the Log-probit model can be considered as acceptable for this example of rather sparse dose–response information. For the use of the AIC criterion, see the section on "Frequentist Combining" below.

A Suite of Models

The identification of one BMDL out of the fit of a suite of models has been a discussion point since the first uses of the BMD approach for the hazard characterization of toxic compounds (U.S.EPA 2012; EFSA 2009; EFSA et al. 2017b), in particular, when the BMDL values of the accepted models varied over a substantial dose range and induced large uncertainty in the determination of the RP/PoD, uncertainty which directly turns into uncertainty of the HBGV. However, in the views of risk managers, a seemingly small difference in HBGVs may have a large impact and might question the applicability of the BMD approach. Whereas a variation of the BMDLs by a factor of 2–3 might be expected form a statistical viewpoint, such a difference could be unacceptable for a HBGV, in particular, when the compound is highly toxic, economically relevant, and/or prevalent in food or environment at nonnegligible concentrations. Model uncertainty, for example, when given by a range of BMDLs, may question selection of models, the specification of acceptance and the procedure of the determination of a final BMDL. Several proposals and concepts have been developed to cope with model uncertainty.

Pragmatic Proposals

The first reactions on the model uncertainty were pragmatic and considered the type and information content of the dose–response data, the type of models judged as acceptable, the degree of variation among the BMD estimates and the BMDL values, and the differences between BMDs and BMDLs. Three aspects were of concern: (i) BMDs and/or BMDLs of acceptable models differ by at least one order of magnitude, (ii) some BMDLs are practical zero or below current detection limits of the toxin, and (iii) the BMDL is substantially lower than the BMD, for example, when the BMD/BMDL ratio is larger than by a factor 5–10 corresponding to a difference of about one order of magnitude. A further discussion point was the width of the BMD-CI for different model.

It was suggested to check each acceptable model by visual inspection of the fit and to assess the shape of the fitted curves in relation to what is expected from biological reasoning. Guidance of US-EPA for BMDs software (U.S.EPA 2012) suggested that a concave shape of the fitted curve at low dose or models with infinite slope at dose = 0 would contradict toxicological knowledge at low doses. Therefore, models with concave shape should be modified by avoiding a steep decline of the response curve when dose tends to 0. Therefore, the model fit was constrained by limiting the range of the slope or other parameters in order to control the shape of the curve for most quantal models (LogProbit, LogLogistic, Weibull, Gamma, and the Multistage- 2) The constraint model replaces so the unconstraint one in situations (i)–(iii).

Another modification of the set of models would be to enlarge the list of Table 1 by allowing both, constraint and unconstraint models for model fitting and define on that extended set a subset of acceptable models, for example, with maximum limits on the allowed differences either among the BMDs or among the BMDLs or between the BMDs and the BMDLs or the distance of the BMDL to the lowest dose tested, see EFSA (2011), Edler et al. (2014), and Davis et al. (2010). Early guidance of EFSA (2009) suggested that BMDLs and BMD/BMDL ratios should not vary by more than one order of magnitude. Otherwise, further examination of the DR data would be recommended and further measures taken, for example, fitting constraint models, changing the size of the BMR. At the extreme, the overall quality of a study would be put on stake and its suitability for dose–response assessment denied. For continuous data, a stepwise decision tree has been proposed by Davis et al. (2010), iterated in the guidance document of U.S.EPA (2012).

It is the Multistage-2 model in the example of sterigmatocystin above where the $BMDL_{10}$ of 0.045 mg/kg b.w. per day was 2–4 times lower than that of other acceptable models. Visual inspection showed a concave curve (Fig. 1d). The constraint model would give a $BMD_{10} = 0.36$ and a $BMDL_{10} = 0.18$ mg STX/kg b.w. per day and fit better to the suite of other BMDLs (Table 1b).

At some instances, it was also argued that a BMDL value should only be used for hazard characterization when it is "within the observed dose-range" by adopting an argument which had been extensively used in the discussion of statistical regression when fitted curves f(x) derived on a bounded region of the independent variable $a \leq x \leq b$ were extrapolated to $x < a$ or $x > b$. Although extrapolation should be handled with caution for hazard characterization, declaring a BMD approach as invalid, when the BMDL is lower than the lowest dose tested, is neither supported by sound statistics nor does it suit the aim of risk assessment to investigate response at low doses – at least as long as the extrapolation does not extend over orders of magnitude. It should be noted that

- With a control group available, the experimental range is from 0 to the largest dose tested and such the BMDL is always in that range.
- As long as the BMD estimate is larger than lowest dose one can hardly claim extrapolation. The BMDL is a lower confidence bound at the statistical confidence level of 95% and depends strongly on that level choice, in addition to dependence on the choice of the BMR value. Both settings might be reconsidered in a discussion of extrapolation.
- Hazard characterization aims for information at the low dose range and can be considered as an attempt for extrapolation by definition. In fact, the regulation problems with cancer risk extrapolation to a risk as low as 10^{-6} and 10^{-5} were one of the reasons to establish the BMD approach.

The limitations of the pragmatic proposals and their different usage by regulatory agencies have stressed harmonization and development of transparent and reproducible approaches as outlined in the next two subsections.

Frequentist Combining

Noting that the BMD approach "does not aim to find the single statistically best estimate of the BMD but rather all plausible values that are compatible with the data" and recognizing the promising results of "multimodel estimation and inference using model averaging" (EFSA et al. 2017b) guidance recognized model averaging (MA) as a method to cope better with model uncertainty. MA has been developed to a useful statistical methodology (e.g., Wheeler and Bailer (2007, 2008, 2009)) such that the revised EFSA opinion from 2017 promoted the use of MA to overcome the diversity of RP/PoD values. Since MA tools were not always available at that time, elements of MA were implemented in a flow chart to determine a BMD-CI. Deviating from previous practice to check acceptability by GoF testing based on the log-likelihood, the AIC values of the single models guided the determination of a BMD-CI, see Fig. 8 in EFSA et al. (2017b). The advice is to fit all models including the *NULL*- and the *FULL-model* and to calculate their AICs for a decision tree. An absolute value of 2 was used to differentiate between AICs and a few decisions guide to a PR/PoD:

- If the all AICs of the suite of models are less than by 2 different from AIC-NULL, the data are rejected, because of no reasonable dose–response relation.
- The model with the lowest AIC is identified and labeled as AIC_{MIN} model. All models with AIC less than by 2 different from AIC_{MIN} (i.e., AIC $\leq$ AIC_{MIN} + 2) constitute the final acceptable models.
- Use the set of models to calculate the BMD-CI and use the smallest BMDL of these models as RP/PoD for the data set and endpoint. Note, however, that small AICs indicate a good fit.
- A criterion for caution checks for $AIC_{MIN} > AIC_{FULL} + 2$ and would stop for seeking further statistical advice when the "best" model might be not good enough compared with the *FULL-model*.
- Identify within a nested model family using log-likelihood method at first the best model and use only this one as acceptable model.

In the example of sterigmatocystin, above was $AIC_{NULL} = 35.30$, $AIC_{FULL} = 35.00$ and $AIC_{MIN} = 30.06$. The critical boundary for the models suitable for the BMD-CI is therefore $AIC_{MIN} + 2 = 32.06$ such that all models except the Probit and Logit model are suitable, including the Multistage2-model with the very low $BMDL_{10}$ of 0.045 mg STX /kg b.w. per day. This model poses therefore still a problem, also in this frequentist approach. It can be resolved when using frequentist MA or Bayesian MA approach that would give a very low weight to this single model.

Model Averaging

The stepwise procedure outlined above is transparent and uses with the AIC an established statistical criterion for model selection. However, it is only a substitute for MA and may be over-conservative by selecting the minimum BMDL. MA combines

the estimates of the different models, not distinguishing between acceptable and non-acceptable models, through a weighted average of the DR models where the weights reflect the relation of the fitted curves to the observed data. The method assumes that the true model is one of the models in the family of models being averaged and it reflects both the sampling variability and model uncertainty. It is expected to yield an RP/PoD higher than the lowest BMDL. A detailed example of MA for quantal data is given in EFSA et al. (2017b). MA has been recommended also in the updated Chap. 5 of FAO/WHO (2009). Even when MA would not yield an acceptable RP/PoD, one can extent the MA to a full Bayesian approach with so-called "soft constraints."

The application of MA for continuous data is less developed than that for quantal data. EFSA et al. (2017b) has only two nested model families in use, the Exponential and the Hill. This and the two options for the distribution of the response (Normal vs. Lognormal) indicate the need for more research on MA for continuous data including means to involve expert knowledge. Note that expert knowledge would also play a role when applying a full Bayesian approach with the need of prior information to run it.

BMD Confidence Interval

The BMD approach accounts for the statistical variability of the dose–response data by calculating statistical confidence bounds. This is an important difference compared with the NOAEL approach. Primarily, statistical uncertainty of the BMD is addressed through its confidence interval ranging from the one-sided lower bound (the BMDL) to the one-sided upper bound (the BMDU). With the default confidence level at 90% (one-sided 95%), the interval (BMDL, BMDU) is a two-sided confidence interval. The lower bound BMDL has been the key descriptor of the BMD approach because of its clear statistical interpretation: When accepting the BMD approach as admissible dose–response assessment method with the BMD as model characteristic (expressible in terms of the model parameters), the specification of a BMR, and when accepting the assumptions made for modeling, the response at the BMDL is with probability 95% not larger than the BMR. The BMDL is therefore in a direct relation with the level of risk specified by the BMR. The BMD estimation is such a basic statistical procedure of dose–response modeling to reach this statement. If the aim is the determination of the BMD, often denoted as the true BMD behind the modeling (i.e. the BMD of the true but unknown model), the BMD-CI is the preferred outcome since it exhibits the complete variability of the BMD and allows a comparison of the uncertainty across models. The BMD-CI covers therefore more uncertainty than the BMDL alone and to some extent it informs on the quality of the design of a study.

BMD Modeling with Several Data Sets

Hazard characterization uses often more than one set of dose–response data for the assessment one critical endpoint of adverse health effects. Most promising for a joint BMD analysis are data based on the same or a similar design such as studies of both

sexes of experimental animals or a sequence of substudies investigating different dose ranges or different generations of experimental animals in studies of reproductive and developmental toxicology. Those data may be found in a sequence of publications of the same group of researchers. Occasionally, dose–response data across species on the same endpoint can be considered together, when they are similar regarding quality and shape of dose–response. A joint BMD analysis of several data sets is possible when a joint common value of the BMR is specified and the same distribution of the response is assumed. The joint analysis of continuous data is simpler when also a common SD is assumed. Different SDs can also be handled, eventually with extra programming.

Two evaluation strategies are possible when several data sets are used:

(a) Perform a separate BMD analysis for each data set as described above, resulting in a corresponding set of BMDs and BMDLs from which an overall RP/POD is determined. Since the BMDLs are numerical values any type of statistical averaging can used, for example, means, medians, or means weighted by a study quality (e.g., width of the BMD-CI). Following the precautionary principle of risk assessment, a low BMDL or the minimum of the BMDLs would be the candidate for the RP/PoD. This precautionary choice is recommended in the case of doubts with combining BMD analyses.
(b) Perform a combined BMD analysis of all selected data sets stratified (i.e., strata are generated) by the factor(s) of the combination such as substudy, sex, etc.

Different from option (a) would (b) perform for each model a joint evaluation of the combined data set with the different strata. For example, the factor sex or other subpopulations can be included as a covariate in the DR analysis (Slob 2002). The statistical analysis checks then whether males and females show significant different DRs or whether the DR data are similar enough to combine both sexes. In practice, one generates, at first, a combined data set with all the available data (doses, number of subjects per groups, incidences if quantal, means and SDs if continuous) and one additional item in the data set for the strata. Next, this combined data set is analyzed accounting for the stratification factor, also called covariate. The statistical analysis is then a general regression with covariates. The methodological advantage of this combination lies in the possibility to have a freedom to define common parameters across data sets when a model is fitted, for example, when a preinspection of the data allows for a common background or a common slope. This reduces the number of parameters and increases the statistical precision of the remaining parameters. Note that the AIC with the number of parameters estimated as one of the two summand decreases with a decrease of the number of free parameters.

Option (b) may be used to combine only a subset of BMD analyses, for example, when using identical parameters. Although there are then less data available, the statistical power of a combined analysis may outweigh that loss by a more precise overall BMDL. Notice that the combined BMD analysis of (b) is different from pooling the data and neglecting the covariate. Pooling data from different data sets

would erroneously ignore the variation across study and could underestimate the risk. Only in the ideal situation when different studies would not differ at all the results should be the same. Overall, covariate adjusted BMD analysis should not be based on statistical reasons only but accompanied by thorough biological and toxicological reasons allowing for the combination of the data sets.

BMD for Human Data and Presence of Covariate Information

The methodology described in the previous section applies equally to human data. More specific guidance for human data has been rare and is mostly available in connections with example data (EFSA 2009; EFSA et al. 2017b). Experimental human data on toxic substances hardly exist because of ethical restrictions, except for few chemicals which had been tested in past for potential therapeutic effects. Hazard characterization with human data is essential for the regulation of many chemical compounds, for example, polychlorinated dibenzodioxins and polycyclic aromatic hydrocarbons (PAHs), methylmercury, cadmium and lead, acrylamide or aflatoxins. However, statistical methods of epidemiology have often been tailored to few pivotal data sets from observational epidemiological studies often addressing a large number of potential endpoints. Those studies are often complex and exposure is usually a continuum from background to occasionally very large doses. Observed doses can also be concentrated within a very narrow range giving not much "room" for the estimation of a dose–response function. Differentiation between possible shapes (e.g., linear vs nonlinear) of the dose–response curve is then difficult or impossible. In addition, human doses have to be estimated, for example, from dietary surveys for food risks in contrast to administered doses in animal experiments. Doses are not under control and may carry substantial error. Human response data do have a large population variation and they are usually confounded by many factors related to personal traits, constitution, behavior, and they may depend on different histories of life-time environment such that competing risk and causality play a big role in the assessment of human data. Recent guidance of FAO/WHO (2009) accounts for the complexity of the modeling of human observational data and addressed as key conditions for the application of the BMD approach:

- Exchangeability
- Positivity
- Consistency

It was noted that the BMD approach as outlined in the previous section needs to be adapted for the lack of a well-defined control group and covariates which may act as confounders. There are also situations, where co-exposure can take the role of a covariate, see, for example, the cadmium and methyl-mercury risk assessment.

A serious restriction of modeling human data is the almost ubiquitous lack of individual data for reasons of privacy and data protection issues up to commercial interests of the data owners. Most data are available for risk assessment only in

aggregated format, for example, percentiles (terciles, quartiles, quintiles, or deciles). This typical granularity raises questions of power and bias compared with the ideal situation of individual data. The BMD approach applied to those aggregated data has to overcome two major hurdles: (i) summary data need adjustment for covariate information and (ii) the specification of a BMR at control is mostly prohibited. A BMR would need to be defined at the background exposure available, for example, at the lowest percentile of aggregated data, possibly including historical information.

BMD Modeling for Different Endpoints

Risk assessment may be based on more than one critical endpoint, and therefore, a multiplicity of endpoints, data sets, and studies may be available for the BMD approach. A prospective planning of the dose–response assessment is therefore recommended to organize the determination of a RP/PoD for the risk characterization. That plan should provide a road map comprising (i) the studies selected (ii) their data sets suitable for modeling, (iii) the various relevant endpoints for the adverse health effect to which (i) and (ii) can be associated, and finally (iv) a prioritization of modeling that accounts for the variety of critical endpoints and data sets available. If possible, a ranking of endpoints by degree of adversity may be helpful. An ordering by size of BMDL or location of the BMD-CI may identify the most critical endpoints if no toxicological arguments would contradict. An exception are genotoxic or directly DNA reactive carcinogens. The BMD approach is then applied separately to cancer incidence as quantal endpoint and MoEs are calculated (EFSA 2005) (see also ▶ Chap. 23, "Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds").

If several endpoints are judged as equally relevant, one may proceed similarly as in the case of several data sets above and summarize them statistically, for example, by a (weighted) mean, median, or the minimum.

Reporting the Results of the BMD Approach

A comprehensive scheme for reporting the outcome of a BMD analysis has been compiled in EFSA (2009) and iterated in EFSA et al. (2017b) with a list of seven items to consider:

1. Summary table of endpoints and their data bases
2. Choice of BMR
3. Software used
4. Assumptions and settings used for modeling
5. Tabulation of the results including all relevant modeling characteristics
6. Graphical presentations of single models and/or outcome of model averaging
7. Conclusion on RP/PoD.

Reporting so-called study BMDLs for all studies identified for the BMD approach, similar as study NOAELs, may apply in special situations but hardly in

complex assessments. In most cases, it might be prudent not to summarize the numerical values into one final RP/PoD for each endpoint, but to report the outcome of all BMD analyses transparently, informing on the quality of the studies from which the data were taken as possible source of variation.

Besides providing information on all endpoints and data sets, it is advisable to justify any decisions made during the BMD analysis, in particular, the specification of the BMR, the model selection, and the specification of the response distribution. Both adherence to and deviation from default assumptions should be justified. Compliance with rules on reproducibility and transparences ask for complete documentation of code and output of the BMD software, usually published electronically as supplementary information.

Use of the BMDL for Risk Characterization

The use of the BMDL as RP/PoD depends on the nature of the critical effect and the mode of action of the effects considered for hazard characterization including also related toxicity endpoints, see ▶ Chap. 23, "Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds." Characterizing the risk of compounds which are both genotoxic and carcinogenic are based on the margin of exposure MOE, the ratio of RP (or PoD) over the estimated exposure dose ED

$$MOE = RP/ED \tag{20}$$

When establishing an HBGV (e.g., an ADI or TDI), uncertainty factors (UFs) are applied to the BMDL in the same way as it was done for the NOAEL approach, see FAO/WHO (2009).

DR data from observational epidemiological studies differ from typical animal toxicity data; however, the BMD approach still applies. A more careful check for the appropriateness of the data is indicated and the influences of confounders on the DR relationship should be addressed for modeling and the interpretation of the HBGV or MoE. The overall UF for human data is usually simpler and smaller since the interspecies extrapolation is dismissed.

The uncertainties of the BMD approach have been addressed above at several instances. Most vulnerable is the preparation of the approach, that is, the selection of the studies, data sets and endpoints and the specification of the BMR. Uncertainty can be reduced by MA techniques or Bayesian procedures, see e.g. the section "Dealing with uncertainty" in Edler et al. (2014). For general guidance on the handing and reporting uncertainty in risk assessment see EFSA (2018).

Cross-References

▶ Do Carcinogens Have a Threshold Dose? The Pros and Cons
▶ Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds
▶ Statistical Evaluation Methods in Toxicology

- ▶ Toxicokinetic Models
- ▶ Toxicological Risk Assessment
- ▶ Uncertainty Analysis in Exposure Assessment-Relevance for Toxicological Risk Assessment

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Human Biomonitoring: Its Importance in Toxicological Regulation

27

Michael Wilhelm

Contents

Abstract

Human biomonitoring (HBM) aims to determine internal exposure to chemicals and related effects. Similar to environmental monitoring, HBM is a basic method for the protection of human health in case of exposure to chemical substances. About 200 chemicals can be analyzed by HBM. In many countries broad general population HBM programs have been established. HBM data are evaluated by

M. Wilhelm (✉)
Department of Hygiene, Social and Environmental Medicine, Ruhr-University Bochum, Bochum, Germany
e-mail: Michael.Wilhelm@ruhr-uni-bochum.de

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reference values and health-based values. Interpretation of HBM data in exposure assessment and health risk context increases its utility and input into risk assessment and risk management.

Introduction

Human biomonitoring (HBM) is defined as the measurement of concentrations of chemicals or their metabolites in human biological media such as blood, urine, or breast milk. Application of hair, pulmonary air, teeth, nails, and saliva in HBM is limited to specific issues. HBM also includes chemical and biological parameters (biochemical effect monitoring, biological effect monitoring) which allow inferences about the pollutants' biological effect. HBM is considered the method of choice for determining internal exposures in the population, population groups, or individuals. Similar to environmental monitoring (EM), HBM is a basic method for the protection of human health in case of exposure to chemical substances. HBM of dose and biochemical effect is an efficient and cost-effective tool to assess human exposure to chemical substances. HBM considers all routes of uptake and all sources which are relevant. HBM is an ideal instrument for risk assessment and risk management. HBM can identify new chemical exposures (merging chemicals), trends, and changes in exposure, establish distribution of exposure among the general population, identify vulnerable groups and populations with higher exposures, and identify environmental risks at specific contaminated sites. The focus of this chapter is on HBM related to environmental and not to occupational exposures. Several overviews on HBM are available (e.g., Angerer et al. 2007, 2011; Needham et al. 2007; Schulz et al. 2011). Figure 1 shows the relation between exposure and health impairment and how EM and HBM are integrated in the scheme.

Environmental Monitoring

Environmental monitoring (EM), also called ambient monitoring, is the determination of chemical substances in environmental samples such as water, air, soil, indoor air, dust, or food (food monitoring). HBM is considered to supplement EM. EM is especially necessary to identify the sources of exposure and to facilitate measures for minimizing emissions. The purpose of EM is similar to HBM to show how well environmental objectives are met and to help detect new environmental issues. The results are also of fundamental importance to environmental management in general, as the drafting and prioritization of environmental policies are based on the findings of EM.

Chemicals (Biomarkers of Internal Exposure)

Nowadays, about 200 chemicals can be analyzed by HBM (Table 1). The number is steadily increasing.

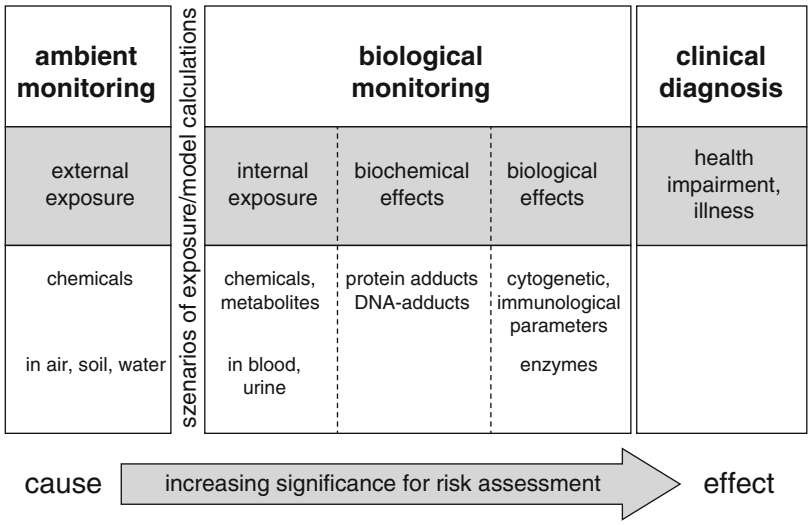

Fig. 1 Relation between exposure and health impairment. (Modified from Angerer et al. 2007)

Biochemical Effect Monitoring

DNA Adducts

DNA adducts are markers of exposure to carcinogenic substances showing the intake of carcinogens and metabolic activation by forming an ultimate carcinogen which can covalently interact with cellular DNA (details in Angerer et al. 2007). DNA adducts represent key events in mutagenesis and carcinogenesis. For the determination of adducted nucleosides, mostly white blood cells and lymphocytes, in some cases sputum and exfoliated urothelial cells, have been used as surrogate tissues. DNA adduct monitoring has been performed in relation to substances like PAH, aromatic amines, dietary heterocyclic amines, and others. Though there are very sensitive techniques for DNA adduct monitoring available, they lack specificity. DNA adducts seem to be a promising tool within molecular epidemiology in population studies; interpretation on an individual level is currently not possible.

8-Hydroxy-2′-deoxyguanosine (8-OHdG)

Besides substance-specific DNA adducts, biomarkers of DNA oxidation are increasingly used in HBM (details in Angerer et al. 2007). Free radicals and other reactive species are constantly generated in vivo and cause oxidative damage to DNA. Oxidative DNA damage is always present and can be physiologically compensated. Additionally, oxidative DNA damage occurs due to exogenous causes, such as inorganic and organic pollutants or their metabolites.

Table 1 Biomarkers of internal exposure in environmental health

Classes of chemical exposure	Chemical, metabolites
Aromatic amines	Aniline, *o*-toluidine, m-toluidine, *p*-toluidine, *o*-anisidine, 3-chloroaniline, 4-chloroaniline, 3,5-dichloroaniline, 4-dichloroaniline, 2-aminonaphthalene, 4-aminobiphenyl
Carbamates	Carbofuranphenol, 2-isopropoxyphenol
Chlorophenols	2-Monochlorophenol, 4-monochlorophenol, 2,4-dichlorophenol, 2,5-dichlorophenol, 2,6-dichlorophenol, 2,3,4-trichlorophenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, pentachlorophenol (PCP)
Disinfection by-products	Bromodichloromethane, dibromochloromethane, tribromomethane (bromoform), trichloromethane (chloroform)
Fungicides	Ortho-phenylphenol, ethylene thiourea, pentachlorophenol, propylene thiourea
Herbicides	2,4-Dichlorophenol, 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, various sulfonylurea herbicides
Metals/metalloids	Aluminum, antimony, arsenic and arsenic compounds/species, barium, beryllium, cadmium, cesium, cobalt, chromium, copper, lead, mercury and mercury compounds/species, molybdenum, nickel, platinum, selenium, thallium, tungsten, vanadium, uranium, zinc
Organochlorine pesticides	Aldrin and dieldrin, oxychlordane, heptachlor epoxide, *trans*-nonachlor, dichlorodiphenyltrichloroethane (DDT), *p,p′*-dichlorodiphenyltrichloroethane (DDT), *p, p′*-dichlorodiphenyldichloroethylene (DDE), *o, p′*-dichlorodiphenyltrichloroethane, endrin, hexachlorobenzene, hexachlorocyclohexane, beta-hexachlorocyclohexane, gamma-hexachlorocyclohexane (lindane), 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, and other pesticide metabolites
Organophosphorus insecticides, dialkyl phosphate metabolites	Diethylphosphate, dimethyl phosphate, diethylthiophosphate, dimethylthiophosphate, diethyldithiophosphate, dimethyldithiophosphate, and specific metabolites: urinary acephate, urinary dimethoate, urinary omethoate, urinary methamidophos
Parabens	Butylparaben, ethylparaben, methylparaben, *n*-propylparaben
Perchlorate and other anions	Nitrate, perchlorate, thiocyanate
Perfluorinated compounds	Perfluorobutanesulfonic acid, perfluorodecanoic acid, perfluorodecanoic acid, perfluoroheptanoic acid, perfluorohexane sulfonic acid, perfluorononanoic acid, perfluorooctanoic acid, perfluorooctanesulfonic acid, perfluorooctanesulfonamide, 2-(*N*-ethyl-perfluorooctane sulfonamido) acetic acid, 2-(*N*-methyl-perfluorooctane sulfonamido) acetic acid, perfluoroundecanoic acid

(continued)

Table 1 (continued)

Classes of chemical exposure	Chemical, metabolites
Phenols	Benzophenone-3, bisphenol A, 4-tert-octylphenol, triclosan
Phthalates	Monobenzyl phthalate, monoisobutyl phthalate, mono-*n*-butyl phthalate, mono-cyclohexyl phthalate, mono-ethyl phthalate, mono-2-ethylhexyl phthalate, mono-(2-ethyl-5-hydroxyhexyl) phthalate, mono-(2-ethyl-5-oxohexyl) phthalate, mono-(2-ethyl-5-carboxypentyl) phthalate, mono-(carboxynonyl) phthalate, monoisononyl phthalate, mono-(carboxyoctyl) phthalate, mono-methyl phthalate, mono-(3-carboxypropyl) phthalate, mono-*n*-octyl phthalate
Phytoestrogens	Daidzein, enterodiol, enterolactone, equol, genistein, *o*-desmethylangolensin
Polyaromatic hydrocarbons	1-,3-,9-Hydroxybenz[a]anthracene; 1-,2-,3-hydroxybenzo[c]phenanthrene, 1-,2-,3-,4-,6-hydroxychrysene, 3-hydroxyfluoranthene, 2-,3-,9-hydroxyfluorene, 1-,2-,3-,4-,9-hydroxy-phenanthrene, 1-hydroxypyrene, 3-hydroxybenzo[a] pyrene, 1-,2-hydroxynaphthalene
Polybrominated diphenyl ethers	2,2′,4,4′,5,5′-Hexabromobiphenyl (BB-153), 2,2′,4-tribromodiphenyl ether (BDE 17), 2,4,4′-tribromodiphenyl ether (BDE 28), 2,2′,4,4′-tetrabromodiphenyl ether (BDE 47), 2,3′,4,4′-tetrabromodiphenyl ether (BDE 66), 2,2′,3,4,4′-pentabromodiphenyl ether (BDE 85), 2,2′,4,4′,5-pentabromodiphenyl ether (BDE 99), 2,2′,4,4′,6-pentabromodiphenyl ether (BDE 100), 2,2′,4,4′,5,5′-hexabromodiphenyl ether (BDE 153), 2,2′,4,4′,5,6′-hexabromodiphenyl ether (BDE 154), 2,2′,3,4,4′,5′,6-heptabromodiphenyl ether (BDE 183), 2,2′,4,4′,5,5′-hexabromobiphenyl (BB 153)
Polychlorinated biphenyls, non-dioxin-like	2,4,4′-Trichlorobiphenyl (PCB 28), 2,2′,3,5′-tetrachlorobiphenyl (PCB 44), 2,2′,4,5′-tetrachlorobiphenyl (PCB 49), 2,2′,5,5′-tetrachlorobiphenyl (PCB 52), 2,3′,4,4′-tetrachlorobiphenyl (PCB 66), 2,4,4′,5-tetrachlorobiphenyl (PCB 74), 2,2′,3,4,5′-pentachlorobiphenyl (PCB 87), 2,2′,4,4′,5-pentachlorobiphenyl (PCB 99), 2,2′,4,5,5′-pentachlorobiphenyl (PCB 101), 2,3,3′,4′,6-pentachlorobiphenyl (PCB 110), 2,2′,3,3′,4,4′-hexachlorobiphenyl (PCB 128), 2,2′,3,4,4′,5′ and 2,3,3′,4,4′,6-hexachlorobiphenyl (PCB 138 and 158), 2,2′,3,4′,5,5′-hexachlorobiphenyl (PCB 146), 2,2′,3,4′,5′,6-hexachlorobiphenyl (PCB 149), 2,2′,3,5,5′,6-hexachlorobiphenyl (PCB 151), 2,2′,4,4′,5,5′,-hexachlorobiphenyl (PCB 153), 2,2′,3,3′,4,4′,5-heptachlorobiphenyl (PCB 170), 2,2′,3,3′,4,5,5′-heptachlorobiphenyl (PCB 172), 2,2′,3,3′,4,5′,6′-heptachlorobiphenyl (PCB 177),

(continued)

Table 1 (continued)

Classes of chemical exposure	Chemical, metabolites
	2,2′,3,3′,5,5′,6-heptachlorobiphenyl (PCB 178), 2,2′,3,4,4′,5,5′-heptachlorobiphenyl (PCB 180), 2,2′,3,4,4′,5′,6-heptachlorobiphenyl (PCB 183), 2,2′,3,4′,5,5′,6-heptachlorobiphenyl (PCB 187), 2,2′,3,3′,4,4′,5,5′-octachlorobiphenyl (PCB 194), 2,2′,3,3′,4,4′,5,6-octachlorobiphenyl (PCB 195), 2,2′,3,3′,4,4′,5,6′ and 2,2′,3,4,4′,5,5′,6-octachlorobiphenyl (PCB 196 and 203), 2,2′,3,3′,4,5,5′,6-octachlorobiphenyl (PCB 199), 2,2′,3,3′,4,4′,5,5′,6-nonachlorobiphenyl (PCB 206), 2,2′,3,3′,4,4′,5,5′,6,6′-decachlorobiphenyl (PCB 209)
Polychlorinated dibenzo-*p*-dioxins	1,2,3,4,6,7,8-Heptachlorodibenzo-*p*-dioxin (HpCDD), 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin (HxCDD), 1,2,3,6,7,8-hexachlorodibenzo-p-dioxin (HxCDD), 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin (HxCDD), 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (OCDD), 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)
Polychlorinated dibenzofurans	1,2,3,4,6,7,8-Heptachlorodibenzofuran (HpCDF), 1,2,3,4,7,8,9-heptachlorodibenzofuran (HpCDF), 1,2,3,4,7,8-hexachlorodibenzofuran (HxCDF), 1,2,3,6,7,8-hexachlorodibenzofuran (HxCDF), 1,2,3,7,8,9-hexachlorodibenzofuran (HxCDF), 2,3,4,6,7,8-hexachlorodibenzofuran (HxCDF), 1,2,3,4,6,7,8,9-octachlorodibenzofuran (OCDF), 1,2,3,7,8-pentachlorodibenzofuran (PeCDF), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF)
Polychlorinated biphenyls, coplanar	3,4,4′,5-Tetrachlorobiphenyl (PCB 81), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), 3,3′,4,4′,5,5′-hexachlorobiphenyl (PCB 169)
Polychlorinated biphenyls, mono-ortho-substituted	2,3,3′,4,4′-Pentachlorobiphenyl (PCB 105), 2,3′,4,4′,5-pentachlorobiphenyl (PCB 118), 2,3,3′,4,4′,5-hexachlorobiphenyl (PCB 156), 2,3,3′,4,4′,5′-hexachlorobiphenyl (PCB 157), 2,3′,4,4′,5,5′-hexachlorobiphenyl (PCB 167), 2,3,3′,4,4′,5,5′-heptachlorobiphenyl (PCB 189)
Pyrethroid pesticides	4-Fluoro-3-phenoxybenzoic acid, *cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid, *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid, *cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid, 3-phenoxybenzoic acid
Tobacco smoke	Cotinine, (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol)
UV filters, benzophenone-type	2-Hydroxy-4-methoxybenzophenone (2OH-4MeO-BP), 2,4-dihydroxybenzophenone (2,4 OH-BP), 2,2′-dihydroxy-4-methoxybenzophenone (2,2′OH-

(continued)

Table 1 (continued)

Classes of chemical exposure	Chemical, metabolites
	4MeO-BP), 2,2′,4,4′-tetrahydroxybenzophenone (2,2′,4,4′OH-BP), 4-hydroxybenzophenone (4OH-BP)
Volatile organic compounds	1,1,1-Trichloroethane (methyl chloroform), 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane, 1,1-dichloroethane, 1,1-dichloroethene (vinylidene chloride), 1,2-dibromo-3-chloropropane, 1,2-dichlorobenzene, 1,2-dichloroethane (ethylene dichloride), *cis*-1,2-dichloroethene, *trans*-1,2-dichloroethene, 1,2-dichloropropane, 1,3-dichlorobenzene, 1,4-dichlorobenzene (para-dichlorobenzene), 2,5-dimethylfuran, benzene, chlorobenzene, dibromomethane, dichloromethane (methylene chloride), ethylbenzene, hexachloroethane, methyl tert-butyl ether (MTBE), nitrobenzene, styrene, tetrachloroethene (perchloroethylene), tetrachloromethane (carbon tetrachloride), toluene, trichloroethene (trichloroethylene), m-/*p*-xylene, *o*-xylene

8-OHdG levels in blood and urine are used in HBM as a biomarker of oxidative stress in relation to exposures to chemicals, physical stress, or tobacco smoking. However, though valid methods for the determination of 8-OHdG are available, diagnostic reliability of this marker is still in debate. It is a marker which is unspecific for the substance taken up. Furthermore, there is a lack of well-established dose–response relations between environmental exposures and the induction of 8-OHdG. Thus, interpretation has to be undertaken with caution, and the biomarker is not suitable for individuals.

Hemoglobin Adducts (Hb Adducts)

Many reactive electrophilic intermediates of mutagenic substances bind to nucleophilic sites of proteins forming protein adducts. The preferred sites are the sulfhydryl group of cysteine, nitrogen of histidine, and N-terminal valine (Angerer et al. 2007). Hemoglobin (Hb) and serum albumin are the preferred monitor molecules because they are readily accessible in large amounts. Considering the life span of Hb (120 days), Hb adducts cumulate in the body. The reaction products of chemical substances with Hb indicate genotoxic properties of that special substance. Since protein adducts are stable and are not removed by active repair processes, they are a more precise HBM tool when compared with DNA adducts. The level of Hb adducts in blood enables the estimation of internal exposure as well as biochemical effects. Examples for chemicals and their adducts are:

- Alkylating agents: ethylene, -oxide → -hydroxyethylvaline, butadiene, -oxide → *N*-(2-hydroxy-3-butenyl)valine, acrylonitrile → cyanoethylvaline, *acrylamide* → *N*-2-carbamoylethylvaline, glycidamide → *N*-(R,S)-2-hydroxy-carbamoylethylvaline
- Nitro aromatic compounds: 2,6-dinitrotoluene → 2-amino-6-nitrotoluene, 2,4,6-trinitrotoluene → 2-amino-4,6-dinitrotoluene, 1-nitropyrene → 1-aminopyrene
- Further examples are Hb adducts for aromatic amines: aniline *o*-toluidine, m-toluidine, *p*-toluidine, *o*-anisidine, 2-aminonaphthalene, and 4-aminobiphenyl

Biological Effects (Biomarker of Effect)

Markers for nephrotoxic effects, such as proteins in urine of subjects exposed to solvents or metals, have been well established. In HBM studies with exposure to genotoxic chemicals, especially the measurement of DNA strand breaks (comet assay) in lymphocytes in white blood cells has become very popular (Angerer et al. 2007). The *comet assay* may be effective in distinguishing exposed from nonexposed groups at high exposure. As with 8-OHdG, the biomarker is not specific; there is still a lack of well-established dose–response relations between exposures and the formation of strand breaks which limits the applicability of this marker in HBM. It may be useful as group indicators, but not for interpretation on an individual level.

Cytogenetic biomarkers currently applied in molecular epidemiologic studies include chromosomal aberrations, micronuclei, and sister chromatid exchange. This important group of genotoxicity biomarkers has been developed in animals, even in vitro, and is now increasingly applied to exposed populations. However, these biomarkers are currently inadequate to HBM purposes, especially for interpretation in individuals.

Other Markers

The concept of individual variability has led to discuss the suitability of *biomarkers of susceptibility*. Of special interest is polymorphism in enzymes such as cytochrome P450 families and the glutathione transferases. Despite the intense work ongoing and the promising results achieved on the pharmacological and toxicological significance of polymorphic metabolizing enzymes, their routine use as HBM biomarkers in environmental health is yet not be validated.

"Omic" technologies include genomics, transcriptomics (gene expression profiling), proteomics, and metabolomics. These new techniques are increasingly utilized in an effort to develop novel biomarkers of exposure, susceptibility, and response to chemicals. The application in the prediction of risks and the prevention of diseases related to chemical exposures is promising, but yet not established in HBM.

General Population HBM Programs

Broad general population HBM programs are established or planned by international, national, and state organizations in a number of countries. One of the most recognized programs is the US *National Health and Nutrition Examination Survey* (NHANES, http://www.cdc.gov/nchs/nhanes.htm). Four surveys have been conducted between 1971 and 1994. In 1999, NHANES became a continuous survey. NHANES includes a physical examination and collecting of biological specimen and a detailed medical history. Approximately 7,000 residents participate each year. Biological specimen is used for clinical and nutritional testing as well as to assess exposure of the noninstitutionalized civilian US population to environmental chemicals. In Germany (Kolossa-Gehring et al. 2012), the nationwide population representative study on exposure to environmental chemicals and its sources comprises of four surveys (*German Environmental Surveys*, GerES I–IV) conducted since 1985 (http://www.umweltbundesamt.de/gesundheit-e/survey/index.htm). GerES IV (2003–2006) was the first survey exclusively on children. A further HBM tool in Germany is the *German Environmental Specimen Bank* (ESB). The ESB is a permanent monitoring instrument and an archive for human species specimens (https://www.umweltbundesamt.de/en/topics/health/assessing-environmentally-related-healthrisks/environmental-specimen-bank). The German HBM activities include the *German Human Biomonitoring* Commission (https://www.umweltbundesamt.de/en/topics/health/commissions-working-groups/human-biomonitoring-commission-hbm-commission). The commission provides general HBM concepts and derives values for interpretation of HBM data since 1992 (Schulz et al. 2011). Other countries with HBM programs include Belgium, Canada, the Czech Republic, Denmark, France, Israel, Japan, and South Korea (for overview see Special Issue, Berlin International Conference on Human Biomonitoring. Int J Hyg Environ Health 215 2012). A more broadly harmonized HBM program has been started throughout the European Union in 2011 (COPHES, http://www.eu-hbm.info/).

Evaluation of HBM Results (Internal Exposures)

Leading concepts for the evaluation of HBM data in the general population have been given by the German HBM Commission (Schulz et al. 2011) as well as by Hays and Aylward (2012).

Reference Values

The German HBM Commission has established the concept of *reference values*. The reference values (RV_{95}) are statistical descriptions of the ranges of concentrations typically seen in a specified reference population but which have no direct

relationship to health effects or risk assessment. They are based on the 95th percentile. The reference values derived by the German HBM Commission for various substances are summarized in Tables 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11. Many data for adults are based on the GerES III performed in 1997–1999. The exposure to most of the substances shown in the following tables has been decreased since then. Striking examples are lead in blood and PCB in blood.

For describing background exposure in the nonsmoking general population to acrylamide (AA) through the acrylamide hemoglobin adduct (*N*-2-carbamoylethylvaline: AAVal) in the blood, the following levels were derived:

- 1.8 µg AAVal/l for nonsmoking children
- 1.2 µg AAVal/l for nonsmoking adults (Schulz et al. 2011).

Hb adduct of acrylamide (AAVal) reflects the acrylamide dose taken up in the previous 4 months.

Reference Value and Risk Assessment

RV_{95} is a strictly statistically derived value and has per se no health relevance. However, RV_{95} is an important tool for prevention to assess whether populations or individuals are more exposed when compared to the environmental background exposure. In case of exposures above RV_{95}, the recommendation is to clarify whether a conspicuous source exists and if it can be avoided. From the perspective of environmental hygiene and preventive medicine, it should be considered whether

Table 2 Gives an overview on HBM guidance values in environmental (nonoccupational) exposures

Value	Basis	Reference
Reference value RV_{95}	Population studies (not always strictly representative)	German HBM Commission
Human biomonitoring value I HBM I	Epidemiological data	German HBM Commission
	Toxicological data	
	Tolerable daily intake (TDI)	
Human biomonitoring value II HBM II	Epidemiological data	German HBM Commission
	Toxicological data	
Biomonitoring equivalent BE	Reference dose (RfD)	Summit Toxicology
	Reference concentration (RfC)	
	Tolerable daily intake (TDI)	
	Acceptable daily intake (ADI)	
	Minimal risk level (MRL)	
	Risk-specific doses (cancer)	

Table 3 Reference values (RV_{95}) for antimony, arsenic, cadmium, lead, mercury, nickel, thallium, platinum, and uranium in urine or blood (Schulz et al. 2011)

Parameter and matrix	Population group (age range)	Study period	RV_{95}
Antimony in urine	Children (3–14 years)	2003–2006	0.3 µg/l
Arsenic in urine	Children (3–14 years)	2003–2006	15.0 µg/l
	Adults (18–69 years)	1997–1999	
Cadmium in urine	Nonsmoking children (3–14 years)	2003–2006	0.2 µg/l
	Nonsmoking adults (18–69 years)	1997–1999	0.8 µg/l
Cadmium in blood	Nonsmoking children (3–14 years)	2003–2006	<0.3 µg/l
	Nonsmoking adults (18–69 years)	1997–1999	1.0 µg/l
Lead in blood	Children (3–14 years)	2003–2006	35 µg/l
	Women (18–69 years)	1997–1999	70 µg/l
	Men (18–69 years)	1997–1999	90 µg/l
Mercury in urine	Children without dental amalgam fillings (3–14 years)	2003–2006	0.4 µg/l
	Adults without dental amalgam fillings (18–69 years)	1997–1999	1.0 µg/l
Mercury in blood	Children who ate fish ≤3 times per month (3–14 years)	2003–2006	0.8 µg/l
	Adults who ate fish ≤3 times per month (18–69 years)	1997–1999	2.0 µg/l
Nickel in urine	Children (3–14 years)	2003–2006	4.5 µg/l
	Adults (not strictly representative)	Not specified	3 µg/l
Platinum in urine	Adults without platinum dental material (18–69 years)	1997–1999	0.01 µg/l
Thallium in urine	Children (3–14 years)	2003–2006	0.6 µg/l
	Adults (20–29 years)	2000–2008	0.5 µg/l
Uranium in urine	Children (3–14 years)	2003–2006	0.04 µg/l
	Adults (not strictly representative)	2001–2003	0.03–0.06 µg/l

Table 4 Reference values (RV_{95}) for chlorophenols in urine of children and adults and pentachlorophenol in serum of adults (Schulz et al. 2011)

Parameter	Population group (age range)	Study period	RV_{95}
2-Monochlorophenol	Children (3–14 years)	2003–2006	7.0 µg/l
4-Monochlorophenol	Children (3–14 years)	2003–2006	15.0 µg/l
	Adults (18–69 years)	1997–1999	
2,4-Dichlorophenol	Children (3–14 years)	2003–2006	2 µg/l
	Adults (18–69 years)	1997–1999	3 µg/l
2,5-Dichlorophenol	Children (3–14 years)	2003–2006	6 µg/l
	Adults (18–69 years)	1997–1999	20 µg/l
2,6-Dichlorophenol	Children (3–14 years)	2003–2006	<0.3 µg/l
	Adults (18–69 years)	1997–1999	
2,3,4-Trichlorophenol	Children (3–14 years)	2003–2006	<0.3 µg/l
	Adults (18–69 years)	1997–1999	
2,4,5-Trichlorophenol	Children (3–14 years)	2003–2006	0.5 µg/l
	Adults (18–69 years)	1997–1999	1 µg/l
2,4,6-Trichlorophenol	Children (3–14 years)	2003–2006	0.7 µg/l
	Adults (18–69 years)	1997–1999	1.5 µg/l
2,3,4,6-Tetrachlorophenol	Children (3–14 years)	2003–2006	<0.3 µg/l
	Adults (18–69 years)	1997–1999	1 µg/l
Pentachlorophenol in urine	Children (3–14 years)	2003–2006	2.0 µg/l
	Adults (18–69 years) living in homes without wood preservatives	1997–1999	5 µg/l
Pentachlorophenol in serum	Adults (not strictly representative)	1995–1996	12 µg/l

Table 5 Reference values (RV_{95}) for metabolites of organophosphorus insecticides (DMP, DMTP, DMDTP, DEP, DETP) in urine (Schulz et al. 2011)

Parameter	Population group (age range)	Study period	RV_{95}
Dimethylphosphate DMP	Children (3–14 years)	2003–2006	75 µg/l
	General population (not strictly representative)	1998	135 µg/l
Dimethylthiophosphate DMTP	Children (3–14 years)	2003–2006	100 µg/
	General population (not strictly representative)	1998	160 µg/l
Dimethyl dithiophosphate DMDTP	Children (3–14 years)	2003–2006	10 µg/l
Diethylphosphate DEP	Children (3–14 years)	2003–2006	30 µg/l
	General population (not strictly representative)	1998	16 µg/l
Diethylthiophosphate DETP	Children (3–14 years)	2003–2006	10 µg/l

Table 6 Reference values (RV_{95}) for metabolites of pyrethroid insecticides (*cis*-Cl $_2$CA, *trans*-Cl_2CA, 3-PBA) in urine (Schulz et al. 2011)

Parameter	Population group (age range)	Study period	RV_{95}
cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (*cis*-Cl_2CA)	Children (3–14 years)	2003–2006	1 µg/l
	General population (not strictly representative)	1998	
trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (*trans*-Cl_2CA)	Children (3–14 years)	2003–2006	2 µg/l
	General population (not strictly representative)	1998	
3-Phenoxybenzoic acid (3BPA)	Children (3–14 years)	2003–2006	2 µg/l
	General population (not strictly representative)	1998	

Table 7 Reference values (RV_{95}) for metabolites of polycyclic aromatic hydrocarbons in urine of nonsmoking children and of nonsmoking adults (Schulz et al. 2011)

Parameter	Population group (age range)	Study period	RV_{95}
1-Hydroxypyrene	Nonsmoking children (3–14 years)	2003–2006	0.5 µg/l
	Nonsmoking adults (18–69 years)	1997–1999	
1-Hydroxy-phenanthrene	Nonsmoking children (3–14 years)	2003–2006	0.6 µg/l/
2/9-Hydroxy-phenanthrene	Nonsmoking children (3–14 years)	2003–2006	0.4 µg/l
3-Hydroxy-phenanthrene	Nonsmoking children (3–14 years)	2003–2006	0.5 µg/l
4-Hydroxy-phenanthrene	Nonsmoking children (3–14 years)	2003–2006	0.2 µg/l
∑Hydroxy-phenanthrene (1,2/9,3,4)	Nonsmoking children (3–14 years)	2003–2006	1.5 µg/l
1-Naphthol	Nonsmoking adults (not representative)		<30 µg/l[a]
2-Naphthol	Nonsmoking adults (not representative)		<20 µg/l[a]

[a]Background exposure values, no strict reference value

this exposure can be reduced as far as reasonably possible. Furthermore, for substances which are considered carcinogenic (genotoxic), no health-based HBM values can be derived; RV_{95} may be also used for risk assessment and risk management. This also applies for other substances for which no threshold is known. For

Table 8 Reference values (RV_{95}) for polychlorinated biphenyls (*PCB*), alpha-hexachlorocyclohexane (*α-HCH*), beta-hexachlorocyclohexane (*β-HCH*), hexachlorobenzene (*HCB*), and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (*DDE*) in whole blood (Schulz et al. 2011)

Parameter	Population group (age range)	Study period	RV_{95}
PCB 28	Children (7–14 years)	2003–2006	0.01–0.1 µg/l[a]
PCB 52	Children (7–14 years)	2003–2006	0.01–0.1 µg/l[a]
PCB 101	Children (7–14 years)	2003–2006	0.01–0.1 µg/l[a]
PCB 138	Children (7–14 years)	2003–2006	0.4 µg/l
	Adults (18–69 years)	1997–1999	0.4–2.2 µg/l[b]
PCB 153	Children (3–14 years)	2003–2006	0.6–3.3 µg/l[b]
	Adults (18–69 years)	1997–1999	
PCB 180	Children (3–14 years)	2003–2006	0.3–2.4 µg/l[b]
	Adults (18–69 years)	1997–1999	
∑PCB (138 + 153 + 180)	Children (3–14 years)	2003–2006	1.0 µg/l
	Adults (18–69 years)	1997–1999	1.1–7.8 µg/l[b]
α-HCH	Children (7–14 years)	2003–2006	<0.1 µg/l
	Adults (18–69 years)	1997–1999	
β-HCH	Children (7–14 years)	2003–2006	0.3 µg/l
	Adults (18–69 years)	1997–1999	0.3–0.9 µg/l[b]
HCB	Children (7–14 years)	2003–2006	0.3 µg/l
	Adults (18–69 years)	1997–1999	0.4–5.8 µg/l[b]
DDE	Children (7–14 years)	2003–2006	0.7–1.4 µg/l[c]
	Adults (18–69 years)	1997–1999	1.5–31 µg/l[c]

Reference values for PCBs, HCB, ß-HCH, and DDT in breast milk (sampled 2003–2005) are 0.5 mg/kg fat for total DDT and ∑PCB (1.64 × (138 + 153 + 180)), 0.06 mg/kg fat for HCB, and 0.07 mg/kg fat for ß-HCH

[a]Reference values had been originally derived related to the detection limit of 0.1 µg/l. Meanwhile, detection limit for PCB 28, 52, and 101 is about 0.01 µg/l. Levels above 0.01 µg/l may indicate an exposure above background exposure

[b]Levels increase between age groups 18 and 69 years continuously. Due to the general decrease of PCB exposure and considering that samples were collected in 1997–1999, the current reference values should be lower at least by a factor of 0.5

[c]Levels increase between age groups 18 and 69 years continuously. Furthermore, data include the comparison between samples collected in West and East Germany. Levels of participants from East Germany were 2–3 times higher compared to those from West Germany

Table 9 Reference values (RV_{95}) for the perfluorinated compounds – perfluorooctanoic acid (*PFOA*) and perfluorooctanesulfonic acid (*PFOS*) in plasma (Schulz et al. 2011)

Parameter	Population group (age range)	Study period	RV_{95}
PFOA	Women, men, children <10 years	2003–2007	10 µg/l
PFOS	Women (not strictly representative)	2003–2007	20 µg/l
	Men (not strictly representative)	2003–2007	25 µg/l
	Children <10 years (not strictly representative)	2003–2007	10 µg/l

Table 10 Reference values (RV_{95}) for aromatic amines in urine of nonsmoking adults (Schulz et al. 2011). The data are based on samples which are not strictly representative. Study period was 2003–2004

Parameter	RV_{95}
Aniline	14.5 µg/l
o-Toluidine	0.20 µg/l
m-Toluidine	0.25 µg/l
p-Toluidine	1.25 µg/l
o-Anisidine	1.10 µg/l
3-Chloroaniline	0.25 µg/l
4-Chloroaniline	1.00 µg/l
3,4-Dichloroaniline	0.45 µg/l
3,5-Dichloroaniline	4.30 µg/l

example, the German HBM Commission recently rescinded the HBM values for lead in blood of children and adults (Wilhelm et al. 2010). For reasons of preventive health protection, the Commission recommends using the RV_{95} for the assessment of lead exposure.

For occupational health purposes, BAR values (Biologischer Arbeitsstoff-Referenzwert) are established by the German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK Commission). These BAR values are similar to the reference values of the German HBM Commission. However, in risk communication two kinds of values with the same meaning may contribute to confusion.

Health-Based Values

HBM Values

The health-related biological exposure limits established by the German Human Biomonitoring Commission are called the *HBM values*. Two levels were defined: the HBM-I value and the HBM-II value. The HBM-I value is a control value, while the HBM-II value is defined as an action level. The HBM-I value describes the concentration in the body matrix of a substance below which no adverse health effect should be expected. At a concentration level higher than the HBM-I and lower than the HBM-II value, an investigation of potential sources of exposure should be undertaken. Exposure to such sources should be minimized, or relevant sources should be eliminated where necessary and achievable with an acceptable level of input. HBM values are derived from toxicological and epidemiological data as well from existing health-based exposure guidance values such as the tolerable daily intake (Table 2). The protection levels intended by the tolerable intake values described above correspond to the protection level intended by the HBM-I value. The HBM-II value describes the concentration in the body matrix of a substance above which relevant adverse health effects may

Table 11 Reference values (RV_{95}) for metabolites of phthalates in urine of children and adults (Schulz et al. 2011). Adults were students from Münster, West Germany

Phthalate	Metabolite	Population group (age range)	Study period	RV_{95}
DnBP	MnBP	Children (3–14 years)	2003–2006	300 µg/l
		Adults (20–29 years)	2006 and 2008	70 µg/l
DiBP	MiBP	Children (3–14 years)	2003–2006	300 µg/l
		Adults (20–29 years)	2006 and 2008	140 µg/l
BBzP	MBzP	Children (3–14 years)	2003–2006	75 µg/l
		Adults (20–29 years)	2006 and 2008	15 µg/l
DEHP	∑5-OH-MEHP +5-oxo-MEHP	Children (3–14 years)	2003–2006	280 µg/l
		Adults (20–29 years)	2006 and 2008	50 µg/l
	5-OH-MEHP	Children (3–14 years)	2003–2006	160 µg/l
		Adults (20–29 years)	2006 and 2008	30 µg/l
	5-oxo-MEHP	Children (3–14 years)	2003–2006	120 µg/l
		Adults (20–29 years)	2006 and 2008	20 µg/l
	5-cx-MEPP	Children (3–14 years)	2003–2006	200 µg/l
		Adults (20–29 years)	2006 and 2008	30 µg/l
	∑3 Metabolites of DiNP	Children (3–14 years)	2003–2006	140 µg/l
		Adults (20–29 years)	2006 and 2008	60 µg/l
DiNP	OH-MiNP	Children (3–14 years)	2003–2006	50 µg/l
		Adults (20–29 years)	2006 and 2008	20 µg/l
	Oxo-MiNP	Children (3–14 years)	2003–2006	30 µg/l
		Adults (20–29 years)	2006 and 2008	15 µg/l
	cx-MiNP	Children (3–14 years)	2003–2006	60 µg/l
		Adults (20–29 years)	2006 and 2008	15 µg/l

DnBP di-*n*-butyl phthalate, *MnBP* mono-*n*-butyl phthalate, *DiBP* diisobutyl phthalate, *MiBP* monoisobutyl phthalate, *BBzP* butyl benzyl phthalate, *MBzP* monobenzyl phthalate, *DEHP* di (2-ethylhexyl) phthalate, *5-OH-MEHP* mono(2-ethyl-5-hydroxyhexyl) phthalate, *5-oxo-MEHP* mono(2-ethyl-5-oxohexyl) phthalate, *5-cx-MEPP* mono(2-ethyl-5-carboxypentyl) phthalate, *DiNP* diisononyl phthalate, *MiNP* monoisononyl phthalate, *OH-MiNP* monohydroxylisononyl phthalate, *oxo-MiNP* monooxoisononyl phthalate, *cx-MiNP* monocarboxylisononyl phthalate

occur, and hence, immediate action to reduce exposure must be taken and expert care in environmental medicine will be required. HBM values are summarized in Table 12.

Table 12 Human biomonitoring (HBM) values derived by the German HBM Commission

Parameter and medium	Population group/age groups	HBM-I value	HBM-II value
Bisphenol A in urine	Children	1.5 mg/l	
	Adults	2.5 mg/l	
Cadmium in urine	Children and adolescence	0.5 µg/l	2 µg/l
	Adults	1 µg/l	4 µg/l
∑Metabolites of di(2-ethylhexyl)phthalate DEHP: 5oxo- and 5OH-MEHP in urine	Children (6–13 years)	500 µg/l	
	Women of reproductive age	300 µg/l	
	Men ≥14 years, general population	750 µg/l	
Mercury in urine	Children and adults	7 µg/l	25 µg/l
		5 µg/g creatinine	20 µg/g creatinine
Mercury in blood	Children and adults	5 µg/l	15 µg/l
Pentachlorophenol in serum	General population	40 µg/l	70 µg/l
Pentachlorophenol in urine	General population	25 µg/l	40 µg/l
		20 µg/g creatinine	30 µg/g creatinine
Thallium in urine	General population	5 µg/l	
∑PCB (138 + 153 + 180) in serum × 2	Infants, children, women of reproductive age	3.5 µg/l	7 µg/l

Biological Equivalents (BEs)

Biomonitoring equivalents (BEs) are defined as the concentration of a chemical or metabolite in a biological medium (blood, urine, human milk, etc.) consistent with defined exposure guidance values or toxicity criteria, including reference doses and reference concentrations (RfD and RfCs), minimal risk levels (MRLs), and tolerable daily intakes (TDIs) (Hays and Aylward 2012). Thus, the definition of BE is functionally similar to the HBM-I value of the German HBM Commission (Angerer et al. 2011). BE values have been derived for more than 80 chemicals (Table 13).

A second BE level has also been defined, the BE_{POD}. This is the BE value corresponding to an exposure level which incorporates uncertainty factors associated with NOAEL or LOAEL, duration adjustment, as well as interspecies extrapolation but which omits uncertainty factors which address intraspecies factors or other database uncertainty factors.

The various HBM guidance values used in occupational health will not be mentioned here.

Table 13 Chemicals for which BE values corresponding to current risk assessment-based exposure guidance values have been derived (Hays and Aylward 2012)

Cadmium	2,4-Dichlorophenoxyacetic acid
Arsenic, inorganic	1,2,3-Trichloropropane
2,4-D Cyfluthrin	Styrene
Deltamethrin	Ethylbenzene
DDT/DDE/DDD	1,2-Dibromoethane
Dioxin TEQ (dioxin, furan, and coplanar PCB compounds)	1,2-Dichloroethane
	Acrylonitrile
Carbon tetrachloride	Toluene
Chloroform	Methyl isobutyl ketone (MIBK)
Hexachloroethane	Furan
1,1,1-Trichloroethane	Tetrahydrofuran
Benzene	*n*-Hexane
Dibromomethane	*n*-Octane
Bromoform	*n*-Nonane
Bromodichloromethane	Hexachlorobenzene
Methylene chloride	1,4-Dioxane
1,1-Dichloroethane	Dibromochloromethane
1,1-Dichloroethene	*n*-Decane
Acrylamide	Tetrachloroethene
1,1,2-Trichloroethane	*cis*-1,2-Dichloroethene
1,1,2,2-Tetrachloroethane	*trans*-1,2-Dichloroethene
Trichloroethene	*n*-Heptane
Bisphenol A	1,1,1,2-Tetrachloroethane
Di(2-ethylhexyl)phthalate	Xylenes, mixed
Diethyl phthalate	Methyl tert-butyl ether (MTBE)
Dibutyl phthalate	Triclosan
Benzyl butyl phthalate	Hexabromocyclododecane
Diisononyl phthalate	PBDE 99

Risk Assessment in Context with Surveys/Tool in Risk Management

Using BEs, hazard quotients are calculated as the ratio of the biomarker concentration to the BE as proposed by Hays and Aylward (2012). *Hazard quotients* <1 indicate that the measured concentration of a chemical in a biological medium is below BE. In this case the exposure is expected to be below the corresponding exposure guidance value. Applying health-based guidance values (HBM-I, HBM-II, BEs) to the NHANES data (geometric mean and 95th percentile population biomarker concentrations) from the report 2012, Aylward et al. (2012) calculated hazard quotients. Most analytes showed hazard quotients below 1. Hazard quotients approaching or exceeding 1 or cancer risks greater than 1×10^{-4} were found for

acrylamide, dioxin-like chemicals, benzene, xylene, several metals, di-2(ethylhexyl) phthalate, and some legacy organochlorine pesticides suggesting that exposure levels may exceed published human health benchmarks. This approach is very useful to assist risk managers in the prioritization of chemicals for more detailed chemical-specific evaluation and risk assessment follow-up.

Cross-References

- ▶ Assessment of Background Exposure and Additional Exposure by Human Biomonitoring
- ▶ Dose-Response Analysis: Identification of Threshold Levels for Chemicals
- ▶ Exposure Scenarios in Toxicology

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Part III

Approaches Used

Toxicodynamic Models

28

Lutz Edler

Contents

Abstract

A toxicodynamic model (TDM) is used to describe a concentration–response relationship primarily for predicting effects at certain concentrations and as explanatory tool for investigating mechanisms of action (MOA) of toxic substances or explaining sensitivity differences between exposed subjects and between species. All such information can specifically contribute to the risk assessment of such substances. Suitable mathematical models and statistical analysis methods have to be applied for the toxicodynamic modeling.

Keywords

Risk extrapolation · Limit risk · Threshold · Point-of departure · Benchmark dose

L. Edler (✉)
Dudenhofen, Germany

Formerly: Division of Biostatistics, German Cancer Research Center-DKFZ, Heidelberg, Germany

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Introduction

TDMs describe the relationship between toxic effects (response) attributed or related to exposure to the toxic substances under investigation or its metabolites and the circulating concentration in the body (or a target organ). Therefore, toxicodynamic modeling is often based on statistical models that relate concentration to adverse effects accounting for biological mechanisms at the site of action (mode of action, MOA) and tissue/cell dynamics. Table 1 informs on the major areas of the application of TDMs and methodological aspects of modeling.

Requirements for Toxicodynamic Modeling

In order to provide information on the MOA of the toxic substance and/or to establish a concentration–response relationship, toxicodynamic (TD) modeling requires careful planning and performing of studies. In general, TD studies may proceed as follows:

- Formulation of the TD issue or question (problem formulation)
- Collection of available preliminary information on MOA and toxicological parameters
- Choice of one or a class of TDMs which appear to fit the purpose
- Selection of the available and appropriate experimental (or observational) data required for modeling
- Identification of model parameters (e.g., known from previous experience) with the potential to inform on the relevant TD characteristic (e.g., maxima or steepness). If applicable screen available data to identify a range of possibly realistic numerical values of the model parameters, when the model fit requires iterative computational procedures and needs such values (so-called initial values) to start the calculation

Table 1 Overview on functions, application areas, and procedures of toxicodynamic models

Function	Application areas	Methods
Describe	Concentration–response curve	Parametric curve fitting
	Toxicodynamic potency	Model fit
	Hypothesis generation	Statistical estimation of concentration (dose) descriptors
Explain	Complex biological and toxicological mechanisms	Biologically based models
	Check or reject hypotheses	Model selection
Predict	Extrapolation to low concentrations	(Non-)parametric curve fitting
	Interspecies extrapolation Intra-species differences in sensitivity	Model fit

- Fit of the TDMs to the concentration–response data and determination (using statistical estimation procedures) of previously unknown model parameters
- Assessing the quality of the model fit
- Interpretation of the estimated parameter values and application of the modeling outcome to the TD question
- Assessing the robustness and the uncertainty of the modeling

An important part of the TD problem formulation is the definition of the TD endpoint (target) and a consideration of its statistical quality in terms of scale of measurement. Three main classes of effect (response) data can be distinguished:

- Quantal measures (e.g., presence/absence of liver toxicity/liver tumors)
- Categorical (ordinal) measures (e.g., degree of impairment of an organ function)
- Continuous (metric, quantitative) measures (e.g., number of tumors in animal experiments, values of an enzyme measured in the blood serum, indices of reproduction).

Besides the statistical information content of a TD endpoint, one should also address the biological information content and toxicological meaning of the TD endpoint. This is, in particular, relevant for the ultimate interpretation of the outcome of the TDM. Therefore, it is important to distinguish between a direct and an indirect target. A direct target would accurately exhibit the effect to be tested in the TD study. Indirect targets (e.g., biomarkers) are applicable when only a surrogate of the target can be measured.

Description and Application of Toxicodynamic Models (TDMs)

Toxicodynamic Potency

Measures of toxicodynamic potency are obtained mainly from descriptive parametric TDMs and their adaptation to concentration–effect curves. Three common TDMs for continuous and two for quantal measures are presented below. All five models allow the determination of a quantity which can be interpreted as TD potency of a substance which expresses an activity often as a descriptor of concentration related to a certain size or degree of a toxic effect.

The Linear Concentration–Effect Model. In cases where a direct proportional relationship between the concentration of the toxic substance C and the effect E can be assumed, the linear model

$$E = E_0 + mC \tag{1}$$

is the simplest model to be applied. E_0 denotes the base effect (background) and m the proportionality of the effect relative to the concentration (slope). The two model

parameters (E_0, m) are statistically determined from concentration–effect data usually available as n data pairs

$$\{(C_i,\ E_i),\ \ i = 1, \ldots,\ n\}$$

where E_i denotes the effect observed at concentration C_i in a total of n observational units (samples) investigated in the TD study. The statistical analysis method is linear regression (see, e.g., Draper and Smith 1998). In practice, this model plays only a minor role since a direct linear relationship rarely applies for TD data. However, the linear model can be used for an interpolation of the concentration–response curve over a few concentrations when only a part of the concentration–effect course is considered, and if linearity can be assumed for that part. Moreover, linear regression is ideal for teaching purposes and for the presentation of the basic concepts of statistical regression and model fitting as sort of statistical reference standard.

The Log-Linear Concentration–Response Model. When the range of concentrations of the TD study is large, covering, for example, several orders of magnitude, one often uses a logarithmic transformation of the concentration in order to visualize the concentration–effect relationship in a graphic when plotting the E_i values versus the $ln(C_i)$ values (ln denotes natural logarithm to the base e). If this results in a graph where E is proportional to lnC, then the log-linear model

$$E = E_0 + m\ lnC \tag{2}$$

with the base effect value E_0 and the slope parameter m can be fitted using the same statistical methods as for Eq. (1). Although the log-linear model is analyzed as the linear model, it should be noted that fitting a log-linear model to data where effects at concentration 0 are included is mathematically more complex. Since the transformed concentration *ln(0)* is for mathematical reasons no more a real number and located outside the range of the plot, at "minus infinity" the fit of Eq. (2) to the TD data can graphically be inspected only at a restricted concentration range. For this reason, the data and the fit are usually retransformed to a linear plot with a logarithmic scale of the abscissa ("x-axis"). It should also be noted that a logarithmic transformation of the concentration may not remove nonlinearity from the concentration–effect relationship. Therefore, one cannot compare the slopes of linear and nonlinear models directly and other nonlinear models (see below) may be investigated. Model fit is obtained again by standard linear regression where for computational reasons the concentration *ln(0)* is replaced by a large negative value to mimic "minus infinity." The choice of this substitute adds uncertainty to the slope estimate. Note that the statistical uncertainty is characterized by a statistical confidence interval with a lower and an upper bound referring to a level of confidence, usually for 90% or 95%.

The Emax Model. Concentration–effect curves, which show a saturation of the effect at high concentrations, exhibit a similarity with curves known from enzyme kinetics and receptor-binding relationships for a long time. They are often evaluated with a model related to the Michaelis-Menten equation. In its simplest form, this so-called Emax model takes the form

$$E = \frac{E_{max} \cdot C}{E_{50} + C} \tag{3}$$

with the two model parameters (E_{max}, E_{50}). Herein, E_{max} is the maximum effect, achieved at the maximum concentration, theoretically at $C = \infty$. E_{50} is the "half-maximum" concentration at which 50% of the maximum effect E_{max} is reached (Yu et al. 2019).

By definition, the effect at background (i.e., at concentration equal to 0) is E_0. In the model Eq. (3), $E_0 = 0$ and such comparable with a linear regression through the origin (0,0) where one is interested in the slope m only. There are two options if the TD study must account for a positive effect at background: (1) one adds a third model parameter E_0 for the background effect such that the model Eq. (3), changes to $E = E_0 + (E_{max}\ C)/(E_{50} + C)$ (response-additive background) or (2) one postulates a virtual background concentration C_0 and replaces on the right side of Eq. (3) the concentration C by $C + C_0$ (concentration-additive background). Practically more important is, however, the extension of the Emax model to the of *Hill type* model, where the concentration C on the right side of Eq. (2) is replaced by an exponentiated expression C^n (the nth power of C) which leads to a sigmoidal shape of the concentration–effect curve. Methods of non-linear curve fitting (regression) are available in standard statistical software packages for the calculation of the model parameters; see, for example, Gabrielsson and Weiner (2016).

Note that the models of Eq. (3) are by one more parameter more complex than the linear and log-linear models since they have two parameters for the shape of the curve compared to only one slope parameter in the linear model. It is obvious that the same class of models can be applied when the toxic effect is also presented on a logarithmic scale. However, the effects will be interpreted on a multiplicative scale in that case.

The Probit Model and Logit Model. Concentration–effect data available as quantal data in a form, where the number of responders r_i and the number of non-responders s_i at the different concentrations (C_i, $i = 1, \ldots, n$) have been analyzed traditionally using parametric models. Therefore, the data are prepared as a set of pairs

$$\{(C_i,\ p_i),\ \ i = 1, \ldots,\ \ n\}$$

where $p_i = r/(r_i + s_i)$ describes the effect as proportion (also called effect rate) at concentration C_i. The *probit model* describes the proportion P as a function of the concentration C by

$$P(C) = \Phi(E_0 + mlnC) \tag{4}$$

with the base effect parameter E_0 and the proportionality (slope) m and where Φ denotes the Gaussian ("Normal") distribution function. A somehow more flexible partner of the *probit model* is the *logit model* which models the likelihood of an effect at concentration C as

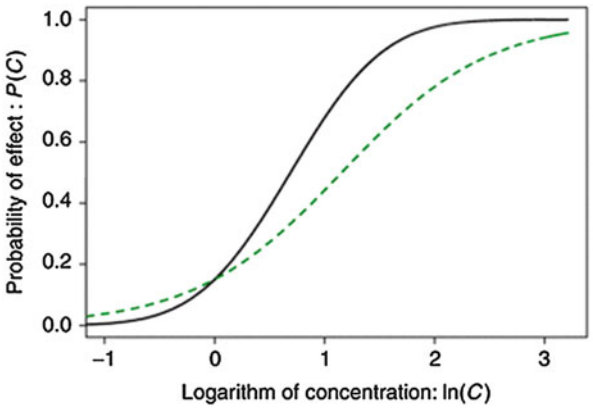

Fig. 1 The probit (*solid line*) and the logit model (*dashed line*) as descriptive TD models. (© Annette Kopp-Schneider, the author thanks for providing the figure)

$$P(C) = \frac{\exp(E_0 + mC)}{1 + \exp(E_0 + mC)}. \tag{5}$$

Applying the linearizing logit transformation one obtains the linear relationship

$$logit\ P(C) = \ln \frac{P(C)}{1 - P(C)} = E_0 + mC$$

and when the concentration is analyzed on logarithmic scale,

$$logit\ P(C) = \ln \frac{P(C)}{1 - P(C)} = E_0 + m \ln C \tag{6}$$

with E_0 as basal effect and parameter m as proportionality factor (see Fig. 1).

Explanatory and Biologically Based TDMs

The model parameters used above express primarily statistical properties of the concentration–effect relationship and therefore these TDMs are often denoted as empirical in contrast to models that integrate biological knowledge of the MOA of a substance in the modeling process, denoted as named biologically based. The latter contain (at least some) model parameters with a physiological or toxicological interpretation and require as consequence, more reliable foreknowledge on the chemical, the biological nature of the toxic substance and its physiological and toxic mechanisms. Consequently, more complex computational modeling is needed for biologically based TDMs, including use of special software when the underlying biological or toxicological features exhibit a higher order of nonlinearity than what is expected for empirical models. On the other hand, fitting a biologically based TD model should always be accompanied with a sensitivity and uncertainty analysis. In

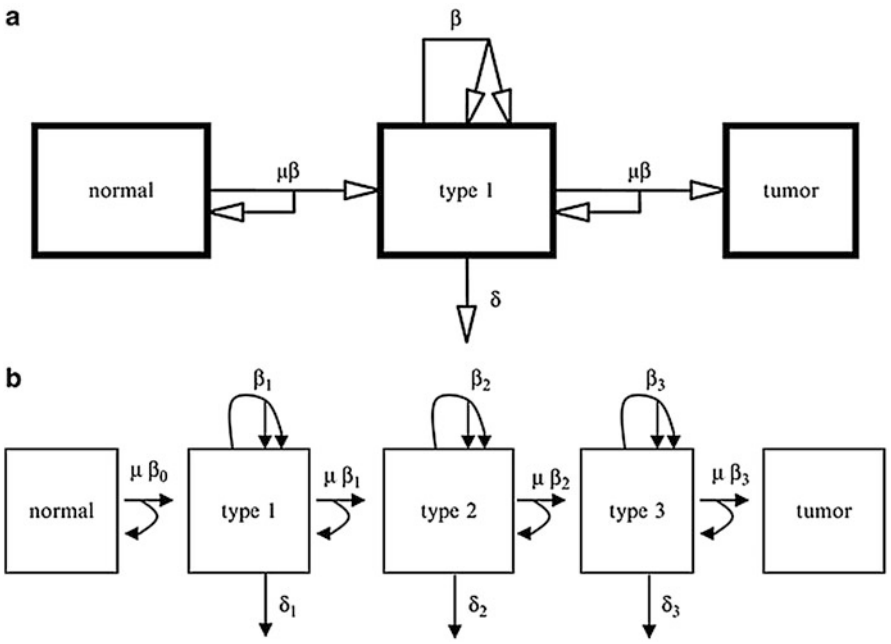

Fig. 2 The two-stage model of clonal evolution of tumors (**a**) and the multistage mutation model of carcinogenesis (**b**, shown here with three intermediates) represent explanatory toxicodynamic models. In the models, μ describes the mutation rates, β the birth rates, and δ the death rates of the cells. (© Annette Kopp-Schneider, the author thanks for providing the figure)

the field of carcinogenesis, the two-stage model of clonal expansion of tumors (initiation-promotion model) and the multistage mutation model (Armitage-Doll model) have been a prominent example of biologically based TDMs (see Fig. 2 and Kopp-Schneider (1997)).

Biologically based TDMs constitute an essential part of biologically based dose–response models (BBDRs) that describe the dose–response from external dose to internal dose and to tissue response. Whereas toxicokinetic-toxicodynamic (TK-TD) models, sketched in the section below, are a subclass of BBDR that has been repeatedly applied in risk assessment, the use of fully developed BBDRs has been rare and limited so far in regulatory context because of their needs in resources and time.

TDMs for Risk Characterization in Regulatory Toxicology

In the context of in regulatory toxicology, risk characterization serves risk managers in decision making (e.g., when deriving regulatory limits). It informs, preferably, in quantitative terms, on the magnitude of risk associated with different exposure levels and helps to establish health-based guidance values (HBGVs). Therefore, risk

characterization synthesizes the two basic lines of risk assessment namely exposure assessment on the one hand and hazard identification and characterization on the other (see ▶ Chap. 6, "Toxicological Risk Assessment"). Thereby the assessments of toxicokinetic and toxicodynamic variability are two relevant steps for establishing a HBGV.

Toxicokinetic-Toxicodynamic Models

TK-TD models combine dose–time relationships of toxicokinetics (TK) with concentration–activity relationships of TD and allow a holistic view of dose-dependent toxic effects over time. TK-TD models are therefore particularly well suited to represent causal relationships between exposure and the toxic effects and thus contribute to a better understanding of the chronologic sequence of toxic effects. Simple TK models can be replaced by physiologically based TK models (PB-TK models) to calculate concentrations in organs and target tissues. From a methodological statistical point of view TK-TD models are directly connected to Pharmacokinetic-Pharmacodynamic (PK-PD) models used in drug research and regulation (Derendorf and Hochhaus 1995*;* Yu et al. 2019).

A further extension of TK-TD models are the TK-TD population models which characterize dose/time–response relationships in populations and combine individual relationships in a comprehensive modeling. They are applicable even to sparse and irregularly sampled individual exposure data, if the sample size is large enough. The statistical analysis is challenging, for example, when using nonlinear mixed effect models or Bayesian hierarchical models. For more details on Bayesian statistical methods and software, see, for example, Lunn et al. (2002).

Chemical-Specific Adjustment Factors

When characterizing the hazard of a toxic substance risk assessors have usually been forced to use dose–response data from experimental animals to derive a Point of Departure (PoD or reference value/reference point RP) using statistical methods (see ▶ Chap. 23, "Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds"). To establish a HBGV for humans exposed to the toxin one needs to extrapolate at first the PoD derived for animals to humans accounting for interspecies differences. Secondly, one has to account for interindividual differences in the human population. Both types of variations (inter-species and intra-species) are accounted using a so-called uncertainty factor UF, formally: HBGV = PoD/UF. A default UF of 100 has been used to convert a PoD derived from an animal study into a HBGV for humans where inter-species and inter-individual differences contributed each the factor of 10. Principles for applying UFs and alternatives allowing data-derived UFs are outlined in FAO/WHO (2009). A specific alternative to default UFs are chemical-specific adjustment factors for substances when sufficient toxicokinetic and toxicodyamic data allow differentiation of sources of uncertainty

such that each of the two factors of 10 is subdivided into a product of two subfactors, one for toxicokinetics and another for toxicodynamics. Defaults were 4.0 and 2.5 for the interspecies differences and 3.2 each for human inter-individual differences for toxicokinetics and toxicodynamics, respectively (Edler et al. 2002). Deviations from those defaults depend on quantity and quality of relevant data. Deviation from the two default TD factors 2.5 and 3.2 can be based on the outcome of TDMs where the factor may reduce or enlarged depending on available data.

Cross-References

- ▶ Do Carcinogens Have a Threshold Dose? The Pros and Cons
- ▶ Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds
- ▶ Toxicological Risk Assessment
- ▶ Uncertainty Analysis in Exposure Assessment-Relevance for Toxicological Risk Assessment

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Toxicokinetic Models

29

Johannes Georg Filser

Contents

Abstract

Toxicokinetics describes by means of mathematical functions the time- and dose-dependent processes of absorption, distribution, and elimination of a chemical substance and metabolites thereof in animals and humans. For this purpose, classical compartment open models and physiologically based toxicokinetic models are used. Both are applied to fit concentration-time data and to predict concentration-time courses of a parent chemical and metabolites usually in blood or plasma for repeated or continuous exposures; the latter models enable additionally to predict such time courses in various tissues and organs. An introduction is given into toxicokinetic modeling exemplified by two classical compartment open models and a physiologically based toxicokinetic model.

Keywords

Toxicokinetics · One-compartment open model · Two-compartment open model · Physiologically based toxicokinetic model · Saturation kinetics · Interspecies scaling · PBTK model · Inhalation toxicology

J. G. Filser
Institute of Toxicology and Environmental Hygiene, Technical University of Munich, Munich, Germany
e-mail: filserjgm@kabelmail.de

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Introduction

Pharmacokinetics and toxicokinetics deal with the mathematical description of absorption, distribution, and elimination of chemicals in animals and humans. Pharmacokinetics focuses preferably on low doses of drugs, and toxicokinetics on both low and high doses at toxicologically relevant levels (Welling 1995). However, there is no strict separation between pharmacokinetics and toxicokinetics in the literature.

Already in 1847, A. Buchanan did some pharmacokinetic calculations on inhalation and exhalation of vaporous diethyl ether. In 1913, L. Michaelis and M. Menten published an equation that described the saturation kinetics of sucrose-invertase activity. This equation is often used in toxicokinetic modeling of enzyme-mediated processes. The origins of pharmacokinetics are generally attributed to the work of E. Widmark and J. Tandberg, who in 1924 described concentration-time courses of "indifferent" narcotics in plasma by means of a one-compartment open model, and to the development of the first physiologically based pharmacokinetic model (PBPK model, also called physiological pharmacokinetic model) published in two papers in 1937 by T. Teorell. In contrast to "classical" compartment open models, PBPK models require physiological and anatomical as well as chemical-specific physicochemical and biochemical data. Since the early 70s of the last century, the development of PBPK models increased continuously with the advances in analytical technologies and in computer software allowing to solve the numerous differential equations that describe these models. Toxicokinetics originated in 1976–1977, when P.J. Gehring and coworkers reported the essential relevance of "pharmacokinetics" in evaluating the results of chronic toxicity/carcinogenicity studies in laboratory animals (Gehring et al. 1976; Watanabe et al. 1977). Physiologically based toxicokinetic (PBTK) models (also named PBT models or physiological toxicokinetic (PT) models) are particularly suitable for such purposes. In the following, the relevance of toxicokinetics for quantitative risk assessment will be shortly elucidated, and the principles of toxicokinetic modeling will be shown on two classical compartment open models and a PBTK model.

The Relevance of Toxicokinetics

Toxicology studies of chemicals in laboratory animals are generally conducted using high doses in order to obtain statistically significant adverse or carcinogenic effects. Often, such an effect is not caused by the chemical itself but by a metabolite. Because the fate of a chemical and its metabolites in the exposed organism is usually characterized by saturable processes, a meaningful extrapolation of the dose-effect relationship to low doses, relevant for human exposure, requires knowledge of the relationship between the external dose (administered amount and exposure concentration, respectively, of the chemical) and the resulting internal dose (body and tissue burdens) of the biologically active toxicant (parent chemical, a metabolite, or both) in laboratory animals and humans. It is obtained by means of toxicokinetic studies. This is why accurate information on chemical-specific toxicokinetics is of utmost

importance for the quantitative assessment of the dose-dependent human health risk arising from the exposure to a chemical when it is based on the results of animal studies. Quantitative risk assessment for a chemical (e.g., the probability of developing cancer for a given scenario of exposure) is generally carried out by national and international agencies responsible for regulating health and safety.

Toxicokinetic Modeling

Toxicokinetics describes by means of mathematical functions the time- and dose-dependent processes of absorption, distribution, and elimination via metabolism and excretion (ADME) of a chemical and of the biologically active toxicant in animals and humans. The required toxicokinetic data are generated in animal studies and, if ethically acceptable, in low dose exposed humans by recording, at diverse doses of the chemical, the resulting concentration-time courses of the chemical and of the toxicant in body fluids and, if a gaseous chemical is dealt with, in the inhaled and exhaled air.

In order to gain species specifically enzyme-related kinetic parameters, dose-dependent concentration-time courses of the chemical and of the toxicant, respectively, are determined in perfused organs, and in an incubation medium, respectively, containing suitable biological material, such as cellular, microsomal, or cytosolic suspensions.

The ADME characteristic parameters are then obtained from fits of a toxicokinetic model to *in vivo* data or from predictions made by a toxicokinetic model that uses *in vitro* data. The most frequently used toxicokinetic models are compartment models. A compartment is defined by its volume. It is well-stirred, which means that the chemical within it is uniformly distributed. The compartments of both classical and PBTK models are open because the chemical enters (input) and leaves (output) them.

Classical Compartment Open Models

Classical compartment open models generally consist of not more than three compartments. The models are data-based: measured concentration-time courses of a chemical in plasma or blood define the number of the compartments to be chosen. For instance, if the distribution of a chemical in the organism occurs too fast for experimental measurement (instant homogenous distribution within the whole organism), a one-compartment open model will be chosen (Fig. 1). If the distribution processes can be observed experimentally, a two- or even a three-compartment open model might be required to fit the data adequately. In most cases, it is sufficient to use a two-compartment open model (Fig. 2). The first compartment, called central compartment, summarizes those organs and tissues that are rapidly perfused (e.g., blood, spleen, heart, brain, kidneys, liver). The chemical concentrations in them are considered to be always in equilibrium with the chemical concentration in the circulating blood. The second, “peripheral”, and the third, “deep”, compartment combine the slowly perfused tissues (e.g., muscles, adipose tissue, skin). They

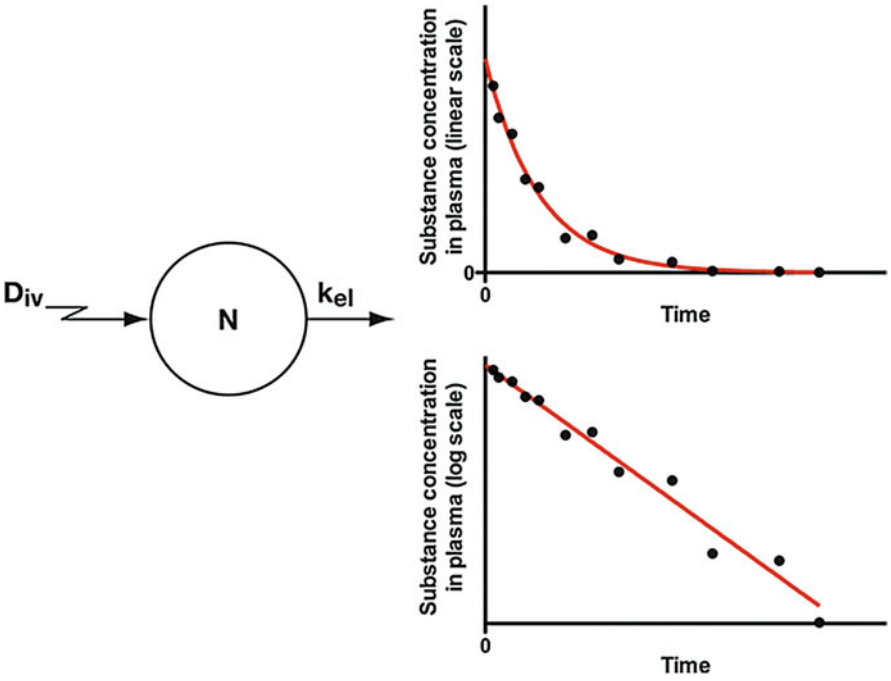

Fig. 1 One-compartment open model for a single intravenous injection of a low dose of a chemical substance that distributes too fast to be experimentally observed, and curve fitted to the measured concentration-time course of the substance in plasma (plotted linearly and semi-logarithmically). Symbols and abbreviations: filled circles, measured data; lines, fitted first-order decay curves; D_{iv}, intravenously administered amount of a substance (dose) at time point zero; k_{el}, first-order elimination rate constant; N, amount of substance at any time point in the compartment. The fitted concentration-time curve is given by $y = y_{(0)} \cdot e^{-k_{el} \cdot t}$. The substance concentration at any time point t is given as y and at $t = 0$ as $y_{(0)}$. The slope of the linear curve in the semi-logarithmic plot represents k_{el}.

require certain periods of time until reaching a kind of distribution equilibrium with the concentration in blood or plasma. The volume of distribution is defined as the ratio of the actual amount of the chemical in the whole organism (except that in the bladder and in the gastrointestinal tract) to its actual concentration in blood and plasma, respectively. Generally, the volume of distribution represents not a physiological space but a fictitious one. Therefore, it is also known as apparent volume of distribution. It is the constant compartment volume in the one-compartment open model. In the two- or three-compartment open models, however, it increases until the end of the distribution phase (see Fig. 2).

At low chemical concentrations, the rates of elimination of a chemical follow first-order kinetics, and the concentration-time course of the chemical in the central compartment is given by an explicit function (see legends to Figs. 1 and 2). By using the rate constants (k_{el}, k_{12}, k_{21}; see Figs. 1 and 2) determined after single intravenous administration of the chemical, predictions of its concentration-time course in

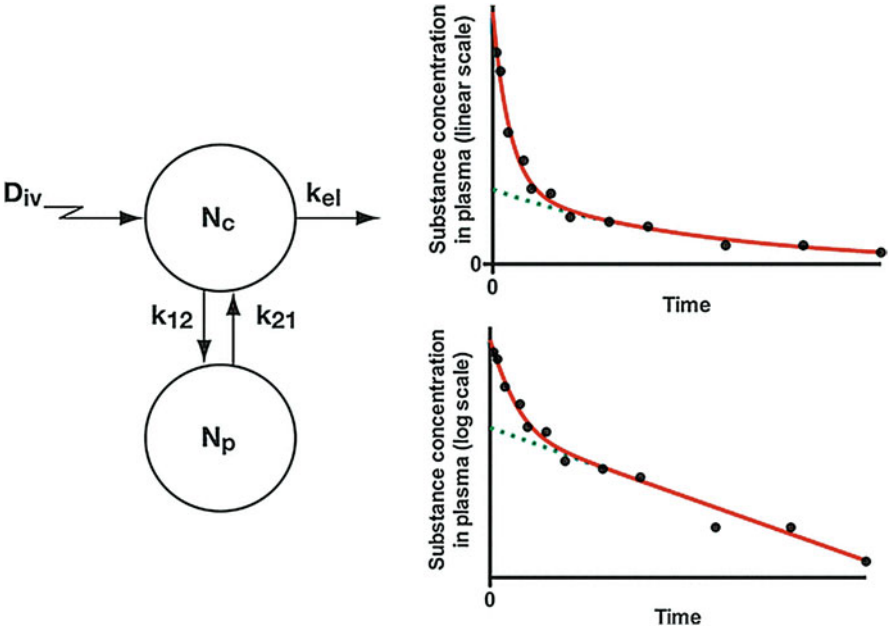

Fig. 2 Two-compartment open model for a single intravenous injection of a low dose of a chemical substance that distributes from the central into the peripheral compartment, and curve fitted to the measured concentration-time course of the substance in plasma (plotted linearly and semi-logarithmically). Symbols and abbreviations: filled circles, measured data; lines, fitted curves; D_{iv}, intravenously administered dose at time point zero; k_{el}, first-order elimination rate constant; k_{12} and k_{21}, first-order rate constants of substance transport from the central compartment to the peripheral compartment and vice versa, respectively; N_c and N_p, amounts of substance at any time point in the central and the peripheral compartment, respectively. The fitted concentration-time curve is given by a function that is composed of two exponential terms: $y = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t}$. The substance concentration in plasma (the central compartment) at any time point t is given as y. The green dotted lines showing the second exponential term of the fitted function have the y-axis intercept B. The constant A is the difference between the initial concentration in the central compartment $y_{(0)}$ and B. The constants α and β consist of the rate constants k_{12}, k_{21}, and k_{el}. The distribution phase is reflected by the first part of the red curve until it meets the green dotted one

blood and plasma, respectively, can be done for repeated or continuous intavenous administrations. With increasing concentration of the chemical, saturation kinetics (e.g., according to Michaelis and Menten) will become evident. Under such conditions, no explicit solution exists for the mass balance differential equations describing the rates of change in the amount of the chemical in each compartment (not shown). A numerical solution has to be used for this purpose.

The disadvantages of the classical models for toxicokinetic issues are evident. No information can be obtained on the target tissue burden of the toxicant. Also, an extrapolation of toxicokinetic parameters between different mammalian species including human (interspecies scaling) is highly problematic because the kinetic information gained by such models has no biological or physiological meaning. These shortcomings are drastically reduced when using PBTK models.

Physiologically Based Toxicokinetic (PBTK) Models

In PBTK models, the compartments correspond to organs, tissues, or lumped groups of tissues with actual, well-defined species- and body weight-specific anatomical volumes. The processes of input and output are driven by the physiological organ- or tissue-specific blood flows and depend on physicochemical parameters (e.g., tissue-to-blood partition coefficients) and additionally on biochemical parameters in metabolizing organs (e.g., a chemical- and organ-specific maximum metabolic rate V_{max} together with the corresponding apparent Michaelis constant K_{map} that is related to the whole organ). Figure 3 shows a flow diagram of a PBTK model for inhalation exposure of a mammal to a gaseous lipophilic chemical that is metabolized in the liver to an amphiphilic, non-volatile metabolite, which is eliminated by metabolism

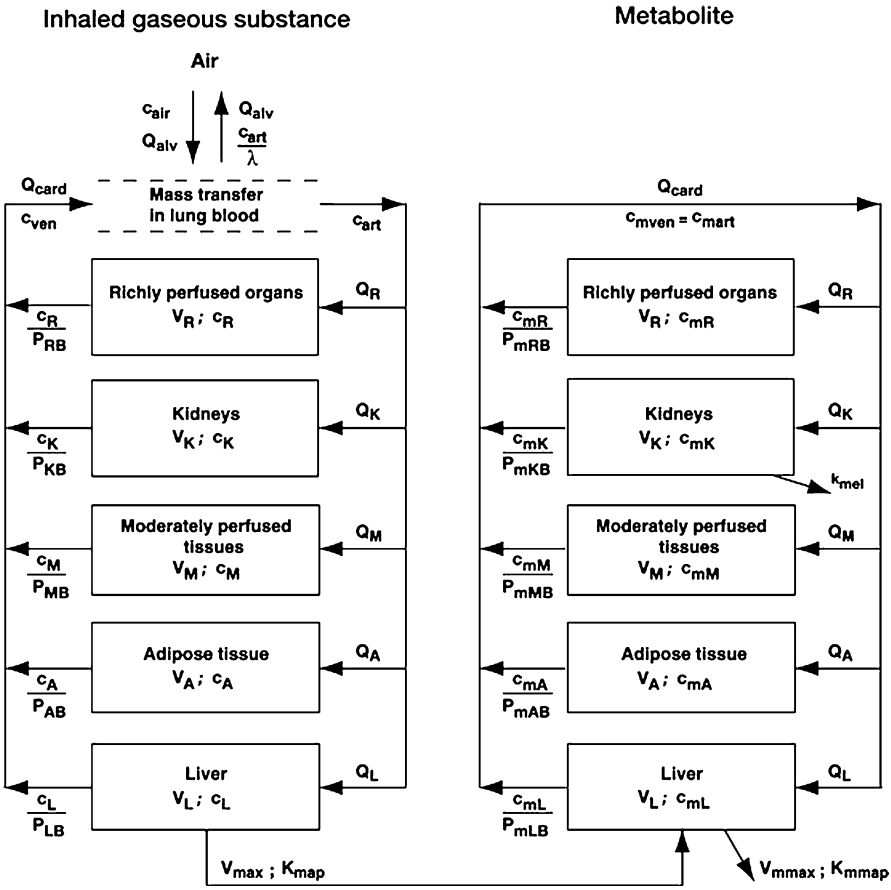

Fig. 3 (continued)

Inhaled gaseous substance

$$\text{(a)}\ c_{ven} = \frac{Q_R \cdot \frac{c_R}{P_{RB}} + Q_K \cdot \frac{c_K}{P_{KB}} + Q_M \cdot \frac{c_M}{P_{MB}} + Q_A \cdot \frac{c_A}{P_{AB}} + Q_L \cdot \frac{c_L}{P_{LB}}}{Q_{card}}$$

$$\text{(b)}\ c_{art} = \frac{c_{ven} \cdot Q_{card} + c_{air} \cdot Q_{alv}}{Q_{card} + \frac{Q_{alv}}{\lambda}}$$

$$\text{(c)}\ \frac{dc_R}{dt} \cdot V_R = Q_R \cdot \left(c_{art} - \frac{c_R}{P_{RB}}\right)$$

$$\text{(d)}\ \frac{dc_K}{dt} \cdot V_K = Q_K \cdot \left(c_{art} - \frac{c_K}{P_{KB}}\right)$$

$$\text{(e)}\ \frac{dc_M}{dt} \cdot V_M = Q_M \cdot \left(c_{art} - \frac{c_M}{P_{MB}}\right)$$

$$\text{(f)}\ \frac{dc_A}{dt} \cdot V_A = Q_A \cdot \left(c_{art} - \frac{c_A}{P_{AB}}\right)$$

$$\text{(g)}\ \frac{dc_L}{dt} \cdot V_L = Q_L \cdot \left(c_{art} - \frac{c_L}{P_{LB}}\right) - \frac{V_{max} \cdot c_L}{K_{map} + c_L}$$

Metabolite

$$\text{(a)}\ c_{mven} = \frac{Q_R \cdot \frac{c_{mR}}{P_{mRB}} + Q_K \cdot \frac{c_{mK}}{P_{mKB}} + Q_M \cdot \frac{c_{mM}}{P_{mMB}} + Q_A \cdot \frac{c_{mA}}{P_{mAB}} + Q_L \cdot \frac{c_{mL}}{P_{mLB}}}{Q_{card}}$$

$$\text{(b)}\ c_{mart} = c_{mven}$$

$$\text{(c)}\ \frac{dc_{mR}}{dt} \cdot V_R = Q_R \cdot \left(c_{mart} - \frac{c_{mR}}{P_{mRB}}\right)$$

$$\text{(d)}\ \frac{dc_{mK}}{dt} \cdot V_K = Q_K \cdot \left(c_{mart} - \frac{c_{mK}}{P_{mKB}}\right) - k_{mel} \cdot V_K \cdot c_{mK}$$

$$\text{(e)}\ \frac{dc_{mM}}{dt} \cdot V_M = Q_M \cdot \left(c_{mart} - \frac{c_{mM}}{P_{mMB}}\right)$$

$$\text{(f)}\ \frac{dc_{mA}}{dt} \cdot V_A = Q_A \cdot \left(c_{mart} - \frac{c_{mA}}{P_{mAB}}\right)$$

$$\text{(g)}\ \frac{dc_{mL}}{dt} \cdot V_L = Q_L \cdot \left(c_{mart} - \frac{c_{mL}}{P_{mLB}}\right) - \frac{V_{mmax} \cdot c_{mL}}{K_{mmap} + c_{mL}} + \frac{V_{max} \cdot c_L}{K_{map} + c_L}$$

Fig. 3 (continued)

in the liver and by first-order excretion in the kidneys. The organism is subdivided into several compartments representing those organs and tissues that are relevant to describe the fate of the chemical and its metabolite. The lung, adipose tissue, liver, and kidneys are represented by their own compartments. The compartment "richly perfused organs" primarily summarizes the brain, spleen, heart, and intestines; the compartment "moderately perfused tissues" mainly represents the muscle and skin. The scarcely perfused bones and cartilage are not taken into account. The model is flow- or perfusion-limited. This means that the rate at which the chemical is transported away from a tissue compartment is limited by the blood flow through it and that the concentration of the chemical in the compartment is in equilibrium with that in the blood leaving it. The mass balance differential equations that describe compartment specifically the rates of change in the quantity of the chemical and its metabolite, respectively, were solved by means of a matching computer software (Berkeley Madonna); tissue-characteristic concentration-time curves of the inhaled chemical and its metabolite were predicted for a short-term exposure during which steady state (the situation in which input and output rates are equal) is not reached yet (Fig. 4).

Many PBTK models for inhalation exposure to gaseous chemicals are more complex, e.g., the model of Filser and Klein (2018), which simulates inhalation exposures of mice, rats, and humans to ethylene, the largest production volume organic chemical worldwide, or to ethylene oxide, a mutagenic and carcinogenic epoxide, which is also of high industrial relevance. The model consists of the compartments lung, richly perfused tissues, kidneys, muscle, adipose tissue, arterial blood, venous blood, and liver. In the liver, ethylene is metabolized to the

Fig. 3 Physiologically based toxicokinetic model for inhalation exposure of a mammal to a gaseous lipophilic chemical substance that is metabolized in the liver to an amphiphilic, non-volatile metabolite (marked by the suffix "m") which in turn is excreted via the kidneys and metabolically eliminated in the liver. Also shown are the equations that describe uptake and elimination processes in each of the physiological compartments. Steady-state equations: concentrations of the substance or of the metabolite (a) in oxygen-poor "venous" blood and (b) in oxygen-rich "arterial" blood. Mass balance differential equations for: (c) the richly-perfused organs, (d) the kidneys, (e) the moderately perfused tissues, (f) the adipose tissue, and (g) the liver. Symbols: c_{air}, concentration of the substance in the air at time t; c_{art} (c_{mart}), c_{ven} (c_{mven}), concentrations of the substance (the metabolite) in the arterial blood leaving the lung and in the venous blood entering the lung at time t; c_R (c_{mR}), c_K (c_{mK}), c_M (c_{mM}), c_A (c_{mA}), c_L (c_{mL}), concentrations of the substance (the metabolite) in the richly perfused organs, kidneys, moderately perfused tissues, adipose tissue, and liver at time t; K_{mapp} (K_{mmapp}), apparent Michaelis constants for the concentration of the substance (the metabolite) in the liver; V_{max} (V_{mmax}), maximum rate of metabolic elimination of the substance (the metabolite) in the liver; λ, substance-specific partition coefficient blood/air; k_{mel}, first-order rate constant of urinary metabolite excretion from kidneys; P_{RB} (P_{mRB}), P_{KB} (P_{mKB}), P_{MB} (P_{mMB}), P_{AB} (P_{mAB}), P_{LB} (P_{mLB}), substance-specific (metabolite-specific) partition coefficients richly perfused organs/blood, kidney/blood, moderately perfused tissues/blood, adipose tissue/blood, and liver/blood; Q_{alv}, alveolar ventilation; Q_{card}, cardiac output (equals the blood flow through the lung); Q_R, Q_K, Q_M, Q_A, Q_L, blood flows through the richly perfused organs, the kidneys, the moderately perfused tissues, the adipose tissue, and the liver; V_R, V_K, V_M, V_A, V_L, volumes of richly perfused organs, kidneys, moderately perfused tissues, adipose tissue, and liver

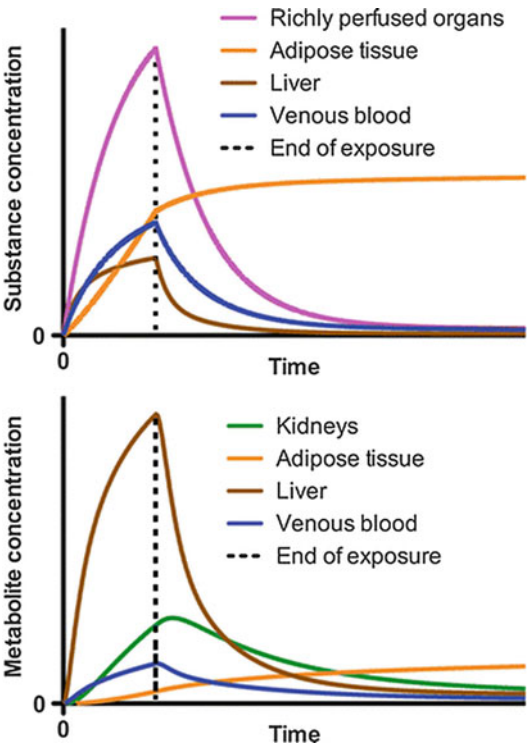

Fig. 4 Predicted concentration-time curves of an inhaled lipophilic gaseous chemical substance and its amphiphilic, non-volatile metabolite in selected tissues of a mammalian organism, generated by means of the physiologically based toxicokinetic model described in Fig. 3. The exposure duration is modeled to be too short to reach steady state. During exposure, the substance is first distributed in the richly perfused organs. The accumulation in the poorly perfused adipose tissue takes place more slowly and is still continuing when the substance concentrations in the blood, liver, and richly perfused organs are already declining due to the end of exposure. The metabolite picture is similar, with the exception that the concentration of the metabolite is high in the liver, the organ in which it is formed. Most of the metabolite peaks are reached after the end of exposure, the latest one in the adipose tissue. The final decreases of substance and metabolite concentrations in the adipose tissue are not shown

biologically active toxicant ethylene oxide by cytochrome P450 2E1, and ethylene oxide is hydrolyzed by epoxide hydrolase to ethylene glycol. In various tissue compartments, including the liver, ethylene oxide is also spontaneously hydrolyzed or is conjugated with glutathione, either spontaneously, or mediated by glutathione S-transferase. For validation of the model, simulated concentration-time courses of ethylene or ethylene oxide in inhaled or exhaled air, and of ethylene oxide in blood as well as predicted levels of ethylene oxide-induced 2-hydroxyethyl adducts in hemoglobin and DNA, were compared with experimental data. The publication contains all the differential equations that describe the model.

PBTK models have the advantage over classical compartment models that they permit knowledge of the fate of a chemical and the biologically active toxicant, respectively, not only in blood or plasma but also in the target tissue of toxicity and in other tissues. They are useful for interspecies scaling of the toxicokinetic parameters of a chemical because sufficient species-specific anatomical and physiological information is usually available. Additionally, the chemical-specific physicochemical and biochemical parameters can be obtained from measurements *in vitro* using animal and human tissues. This is why the use of such models for risk assessment purposes is continuously growing. However, it has to be stressed that interspecies scaling easily results in erroneous predictions on the blood and tissue burdens of metabolites. Model predictions should always be treated with caution as long as they are not validated by a comparison of predicted data with species-specific experimental *in vivo* data.

Cross-References

- ▶ Aims and Mission of Regulatory Toxicology
- ▶ Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds
- ▶ Importance of Xenobiotic Metabolism: Mechanistic Considerations Relevant for Regulation
- ▶ Toxicodynamic Models
- ▶ Toxicokinetic Tests

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Exposure Scenarios in Toxicology

30

Gerhard Heinemeyer

Contents

Abstract

Exposure assessment represents, besides hazard identification, the second pillar that is needed for risk characterization. Exposure assessment attempts to quantify human exposures to potentially toxic substances by considering the relevant parameters: source, amount, site/place, frequency/duration, pathway/route, and

G. Heinemeyer (✉)
Formerly German Federal Institute for Risk Assessment, Berlin, Germany
e-mail: gerhardheinemeyer@o2mail.de

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human activity. The external exposure of the pulmonary, dermal, or gastrointestinal surfaces will – when absorption takes place – result in respective internal exposure of the organism. Exposure scenarios use the best available parameter information and apply it to a specific problem, e.g., an increased indoor air concentration of a solvent, to estimate the potential risk for different populations, such as children or aged people, taking behavior and anthropometric data into account. Deterministic and probabilistic models for quantitative exposure estimates are in use. Variability and uncertainty should be taken into account. The basics of this methodology and regulatory aspects are described in this contribution.

Keywords

Risk characterization · Exposure source · Exposure pathway · Exposure route · External exposure · Internal exposure · Exposure scenario · NOAEL · Exposure modeling · Exposed population

Introduction

Exposure is defined as the

> concentration or the amount of a particular agent that reaches a target organism, system, or (sub)population in a specific frequency for a defined duration. (WHO/IPCS 2004)

Exposure can be understood as dose estimation, by the oral, dermal, or inhalation route and is normally characterized by means of exposure scenarios. The information from the exposure scenario is needed for building up an exposure model. Exposure models can be understood as a translation of an exposure scenario to a mathematical algorithm to yield a qualitative and a quantitative estimate of exposure.

Exposure assessment is based on three basic elements: (i) the exposure scenario, (ii) the exposure model, and (iii) the exposure parameters[1] (WHO/IPCS 2005). The basic characterization of the exposure is made by the exposure scenario (ES). The ES describes the circumstances of exposure, covering all situations and corresponding information needed to perform an exposure estimate. The WHO (2004) defines the term exposure scenario as

> a combination of facts, assumptions, and interferences that define a discrete situation where potential exposures may occur. These may include the source, the exposed population, the time frame of exposure, micoenvironment(s), and the activities. Scenarios are often created to aid exposure assessors in estimating exposure.

This definition should be used as a basic concept for exposure estimation.

Since 2006, an additional definition of exposure scenario must be taken into consideration regarding to the European Chemicals Regulation (REACH; European Commission 2006). In the regulation, the exposure scenario is defined as

> . . .the set of conditions that describe how the substance is manufactured or used during its life-cycle and how the manufacturer or importer controls, or recommends others to control, exposures of humans and the environment.

This definition is basically not different to the above one, but its focus is the characterization of the measures to control the exposure and the measures to reduce it, where needed.

This chapter is explaining the exposure scenario on the basis of the WHO definition, with hints of the particularities of the REACH regulation.

Similar to drug treatment, an exposure estimate can be understood as the dose of a contaminant or hazardous substance that can be taken in by an individual or a population.

Structure of Exposure Scenarios

Exposure scenarios describe the complex characteristics of the external exposure from any substance that can be released from a variety of sources, e.g., the environment, consumer products, food, and other sources. The resulting external "dose" will be systemically absorbed and results in the toxicologically relevant "internal" exposure. The characterization of the exposure scenario describing external exposure should be divided into several parts to be combined with each other yielding the complete scenario. The different elements of an exposure scenario are broadly characterized in Heinemeyer (2019a) as shown in Fig. 1.

In the REACH regulation (European Commission 2006), the scenario contains basically the same information. However, its focus is on the information which measures are considered by the producer of the product to control the exposure to an extent that will not exceed the derived no-effect level (DNEL). If, for example, in an

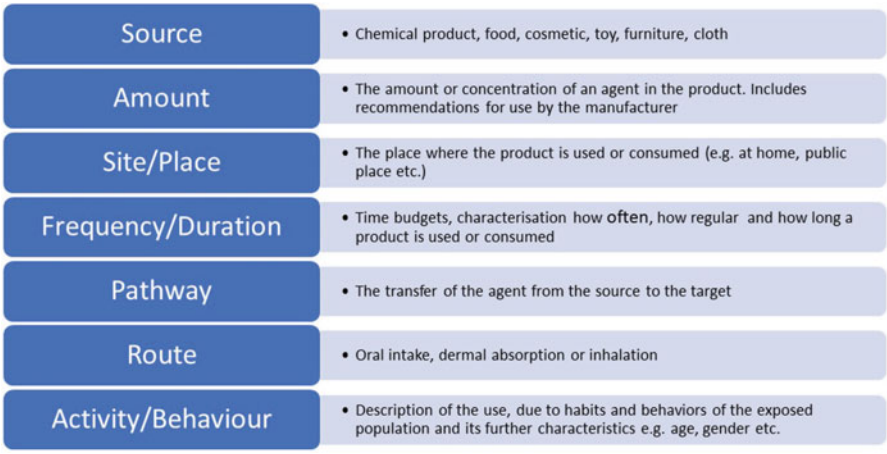

Fig. 1 Elements of an exposure scenario

exposure calculation, the DNEL is exceeded, the registrant[1] must implement measures to reduce the exposure by risk management measures (Bruinen de Bruin et al. 2007). Examples for RMM are reduction of the concentration of a substance in a product, hindrance of migration of a substance from an article, or release reduction by special dispensers. Non-exceedance of the DNEL indicates that a product is safe.

A scenario can be characterized by three parts (subscenarios). There may be an overlap of the above-shown elements of the scenario.

The first part of the exposure scenario describes the source of the substance of interest. This source may be a chemical product, food or any other products used by consumers or workers. Water, air, and soil may also serve as source. This subscenario covers information about the amount of an agent in that source that will be potentially released. The kind of the source also determines frequency and the duration of the release.

Example
A certain household cleaner (source) containing a substance in a particular concentration will be applied to a bigger area (e.g., the ground of a room) and released during and after application.

The second part of the exposure scenario describes the release and transfer from the source (pathway of exposure) and its contact and with the exposed person and the following intake/absorption (route of exposure).

Example
The substance, due to its vapor pressure and molecular weight, will be released and evaporated to room air to yield a certain concentration. The concentration will increase and continue over time and can be inhaled by persons in that room. Due to air exchange, the concentrations in room air will decrease. Substances having low volatility will be distributed mostly via the house dust path.

The third part of the exposure scenario describes the use, due to habits and behaviors of the exposed individual or population. This includes the information about activities of people using that products The frequency and duration of the actual use (working with a product, eating food, etc.*) is considered. The information is often scarce and thus is mostly characterized by default values.*

[1]Registrant: The company that prepares the chemical safety report for notification to ECHA (European Chemicals Agency)

Example 1
The exposed person stays in that room for a certain time and will inhale the air. The time an exposed person is spending in a room may account for, e.g., 4 h.

Example 2
An individual or a population eats a combination of food over a certain time period and with different frequencies.

Characterization of the Source and the Use of the Substance in a Product

This subscenario is used to characterize the source of the substance and the amount that is potentially released during the use of the product. The limitations of these processes are determined by the product itself that contains the substance, its physicochemical properties, its concentration, and the mode of use.

Categories of Use

A substance may appear as an ingredient in many different products and product types (Heinemeyer and Hahn 2005). An approach that characterizes product use categories can therefore be very helpful to identify the sources of substances. Product categories have been used on the national and international level. Some of the documents became "official" due to their use in technical guidance documents (ECHA *2010*) or from use and recommendations by international agencies (EFSA 2009) and organizations (WHO *2005*). Therefore, they have some standardizing character, although the details are differing. Major importance is due to the guidance documents and classifications used in international databases, such as the *industrial categories* and *product and article categories* described in the ECHA guidance R12 *use descriptor system* (ECHA *2010*) (Table 1).

The impact of classification of products has recently been described by Heiland (2019). Also, poison centers around the world are using product use classification systems for documentation of cases and to prepare annual reports. In most of the classification systems, a differentiation is made according to the use of the products, e.g., paints, household cleaners, pesticides, cosmetics, and others. Due to these documentations, it can be checked how close exposure scenarios are close to reality (Heinemeyer and Hahn 2005). The identification of use of a substance and the description of manufacturing and the use process is an important part in defining exposure scenarios under REACH (van Engelen et al. 2007; Heinemeyer 2008).

Table 1 Important sources of information on classification systems to characterize exposure scenarios

Reference	Editor	Remarks
AUH report	Behörde für Arbeit, gesundheit und Soziales, Hamburg. **A**usschuss für **U**mwelt**h**ygiene der AGLMB	Food intake data from the national survey 1985–1989
Bundeslebensmittelschlüssel	BVL (2012a) and Max-Rubner Institut (2012)	Nutrient database with food category system, national, Germany
Food contamination surveys	For example, BVL (2012), EFSA (2009)	
EFSA concise food consumption database	EFSA (2012)	A collection of national food consumption data due to harmonized food grouping
EFCOSUM report	Efcosum Consortium (2001), Brussard et al. (*2002*)	Report from an EU research project
LanguaL	Møller and Ireland (2010)	
EIS-Chemrisks	EU Commission, Joint Research Centre, Ispra	Project report and database EIS–Chemrisks
GEMS food	WHO (2012)	Worldwide classification system for foods
ECHA technical guidance document R12	European chemicals agency (*2010*)	Compilation of different product and article categories and product use classification for REACH
EU commission	Technical guidance document 2003	
General factsheet	RIVM; Bremmer et al. (2006a)	Collection of exposure defaults and assumptions
Paint products factsheet	RIVM, Bremmer and van Engelen (2007)	Collection of model parameters for paints
Pest control products factsheet	RIVM, Bremmer et al. (2006a)	Collection of model parameters for pesticides
ECETOC TRA	European center for ecotoxicology and toxicology of chemicals, several versions (*2012*)	Guidance document and tools for targeted exposure assessment
Annual reports of poisonings reported due to chemical law	Federal Institute for Risk Assessment (2011)	Product classification developed on national levels in cooperation with poison centers
INTOX	WHO (2012)	Classification developed for poison center annual reports

The development of classification systems available for foods is more advanced than those for other products mentioned above. Food classification was developed since longer times for systematic characterization and for nutrition evaluation, e.g.,

for food consumption surveys. The data are also used for exposure assessments. In the EU, the European Food Safety Agency (EFSA) has introduced a harmonized food classification characterization in its *Comprehensive Food Consumption Database* (EFSA *2012*), which comprises data on food consumption from nearly all EU member states. In Germany, the *Bundeslebensmittelschlüssel* (BVL *2012a*; Max-Rubner-Institut 2012b) is used to classify food. The Max-Rubner Institute is responsible to maintain this classification system up to date which is close to the *LanguaL* (Møller and Ireland 2010). The latter combines a fixed three-level thesaurus with relational and dynamic tables, so-called facets. Product/use categories can be transferred and expressed as subscenarios on different levels of aggregation to apply a standardized approach (use model) with respective exposure (model) parameters. Food classification is also extensively described by Fabiansson (2019).

Release, Distribution, and Disappearance

As in pharmacokinetics, this part of the exposure scenario describes the appearance, distribution, and disappearance of a substance in an environment. This subscenario regards mainly for exposures via the air and inhalation. It includes:

1. The description of the concentration of the substance (as described above) in the product and its release, by migration, evaporation, or emission.

2. The distribution of the substance in the surrounding environment to which emission occurs, either bound to particles, e.g., house dust, or in the gas phase.

3. The disappearance of the substance from the environment.

Source, (micro)environment, and substance characteristics are limiting the release of the substance. In combination with the use, the route of exposure will be oral, dermal, or by inhalation. In some exposure scenarios, source, pathway and route are characterized by one process, e.g., exposure via food consumption.

Exposure by Inhalation

The scenario characterizing the exposure by inhalation normally describes the concentration – time course of a volatile substance in the indoor air, either in one or multiple rooms. The concentration can be used for comparison with toxic concentrations.

It is recommended to use the concentration in air to estimate the uptake of a substance via the lungs to the systemic circulation by using the inhaled air volume and an absorption factor. Internal exposure evaluation enables risk assessors to

estimate total body burden, e.g., in children or other particular populations. To perform these estimates, the respiratory volumes per time and pulmonary absorption rates are needed.

In addition to inhalation of substances in the gas phase, the inhalation of small particles should be also taken into account. Dust is a vehicle for nonvolatile substances that can be adsorbed and desorbed from the particles, absorbed through the alveolus, and thus enter the human body.

Exposure parameters needed to estimate exposure from inhalation

- Concentration of the substance in room air
- Concentration of the substance associated with fine dust particles
- Migration rates (release rate per time)
- Vapor pressure
- Molecular mass
- Density
- Product amount used in the application
- Concentration of the substance in the product
- Duration of the application
- Room volume
- Air exchange rate
- Inhalation volume per time
- Absorption factor

Typical Scenarios of Inhalation Exposure

1. Use of volatile substance, e.g., solvents in paints, laquers, or cleaners

A certain amount of a product (e.g., a paint or cleaner) will be applied to a surface. A volatile substance will be evaporated and produces indoor room concentrations. The substance distributes in the room and disappears after some time, according to the air exchange rate. This type of scenario has been considered, e.g., in the computer tool ConsExpo (RIVM 2017) and the wall paint emission model published by the US EPA (*2001*).

2. Emission from solid bodies

A constant amount will be evaporated over a longer time period from, e.g., furniture and textiles. This may lead to constant (steady state) concentrations of the substance in indoor air. The extent of this concentration depends on the air exchange rate, temperature, and other factors, e.g., whether the substance can be adsorbed to particles. This scenario may be applicable for exposures from inhalation due to solvent contaminated residual wastes.

3. Inhalation of dust

Dust inhalation represents a special form of exposure by inhalation. By this pathway, inhalable fine particles from the microenvironment that can adsorb substances enter the lungs and alveolus. After desorption from the particles, the substance can be absorbed to the systemic circulation. Sometimes, they can remain in the alveolar cells and lead to local effects, as particles as well. The concentrations in the dust cannot be estimated and have to measured.

The example shown below represents an estimation of exposure for a child (bodyweight (BW) 8.10 kg). This estimate is characterized by its conservatism, taking low body weight (fifth percentile), a respiratory volume (RV) that considers (partly) activity *and* rest, as well a maximal contact time (CT) and a high pulmonary absorption rate (RPA; 100%). This approach is often called "worst case."

Example: Conservative Estimation of Exposure by Inhalation

- Concentration in room air (estimated or measured, C) – 10 μg/m ^{3}
- Body weight (BW) – 8.1 kg
- Respiratory volume per time (RV) – 2.9 m ^{3}/day
- Contact time (TC) – 1 day
- Pulmonal absorption rate (RPA) – 1
- Inhalation exposure (absorbed amount) [C*RV*RPA*TC/BW] – 3.5 μg/kg/day

Dermal Route of Exposure

The dermal exposure estimation characterizes the amount of a substance which is on the skin and can be absorbed through the skin.

Typical Scenarios of Dermal Exposure

1. Use of cosmetic products

A product will be applied to skin; one or more substances in the product can be absorbed through the skin. In dermal exposure assessment, products that can remain on skin (nonrinse) will be differentiated from those that will be removed by washing (rinse off).

2. Use of household cleaners

The hands will be shortly put into the water that contains the washing product. Substances in that diluted product can be adsorbed to and remain on skin and may be dermally absorbed. When taking a bath, the whole body surface will be exposed.

3. Dermal exposure via air

Volatile substances in the air can come into contact with the skin and are dermally absorbed. Normally, the extent of this exposure is small.

4. Wearing textiles and contact with leisure and hobby products

Direct contact of substances from textiles or leisure and hobby products with the skin is possible by migration to the skin. The exposure surface is the part of skin that is covered by the textile or contacting the leisure and hobby product.

5. Contact with pets

Ingredients from, e.g., pesticides used for domestic animals to treat against pest may lead to dermal contact when touching pets. Children may have oral exposure after licking hands (mouthing behavior) after touching the animals.

A basic rule for estimating dermal exposure has been described in the EU technical guidance document for existing chemicals and has been taken over by ECETOC (2005) as well as in the ECHA technical guidance documents (*2015, 2016a, 2016b*). The *amount* (AM) that can lead to exposure can be estimated from the *area* (A) of exposure times an estimated *thickness of the layer* (TL) of 0.01 cm (default value) and from the *concentration* (C) of the substance in the *product* (AM = A*TL*C). In some documents, additional *absorption rates* given as percentages are used. However, it must be considered that dermal absorption is a time-dependent process. Taking percentages as rates can lead to errors and should only be applied as a default assumption, e.g., a conservative concept assuming 100% of absorption through the skin. For short contact times (e.g., shortly applied cosmetics), correction factors have been introduced that reduces the absorption rate. In general, values from 1 – (10) – 50% are used as default assumptions, with different justifications, depending on the purpose of the evaluation. For some substances, absorption constants and coefficients have been derived, due to lipid solubility (octanol/water coefficient) and molecular weight. Respective models have been established by Wilschut et al. (1995) and have been integrated into the ConsExpo tool (RIVM 2017).

Exposure parameters needed to estimate dermal exposure.

- Exposed skin area (e.g., 840 cm^2 for hands)
- (Theoretical) thickness of layer (0.01 cm; mixtures; 0.001: articles)
- Concentration of substance in the product
- Migration rates of the substance (measured)
- Absorption coefficient (derived by model evaluation), alternative: absorption rates (conservative estimates, percentages)

Oral Route of Exposure

Oral exposure characterizes the oral intake of a substance by mouth and the amount that is absorbed in the gastrointestinal tract. Oral intake is possible with food, drinking water, house dust, the mouthing behavior, and some personal care products (e.g., tooth paste). House dust and related paths are particularly important in small children. In general oral exposure estimation requires knowledge of the concentration of the substance in and the amount of the medium that is taken in.

Typical Scenarios

1. Intake of food and drinking water

A number of different sources have to be distinguished to estimate the dietary exposure to contaminants in the food chain, pesticide residues, food additives, process contaminants, substances in food packaging, and bacterial toxins and metabolic products. Process contaminants, e.g., acrylamide or MCPD, (3-Chlor-1,2-propandiol) can be formed during heating of foods.

Dietary exposure estimation is normally performed by multiplying concentration data in the food and the respective food consumption data and correction factors, e.g., for storage and preparation. Concentrations in food can be obtained from, e.g., market control measurements. However, as these data are risk oriented, there is a reason for expecting high concentrations. Systematic and representative evaluations of concentrations in food are more adequate to study dietary exposure in a population. Such data are available from, e.g., the German food monitoring system (BVL 2012). The European Food Safety Agency is establishing a system to regularly collect data of concentrations of substances in food, collected from the member states (EFSA 2011). Due to the immense number of samples needed to describe concentrations in food, approaches have been developed to reduce numbers of sample by, e.g., pooling, for example, by the concept of total diet study (TDS; Blume and Lindtner 2019).

The identification of food consumption data normally is performed by means of questionnaire studies. On the national levels, food consumption surveys have been performed in many countries, for example, the "Nationale Verzehrsstudie II" (Max-Rubner-Institut 2012a) in Germany. There are several methodological approaches by which consumption studies can be performed (24-h recall, dietary history, food frequency study, diary studies, with and without weighing the food) (Straßburg 2019). It should be mentioned that these study types have advantages and disadvantages for the particular questions asked in risk assessment, e.g., acute or chronic hazards (Lindtner and Heinemeyer (2019).

To perform food consumption studies, foods will be characterized by a food basket that contains a significant part (normally >90%) of all foods eaten. The particular foods should be classified by a systematic food group classification system (see respective chapter).

Food exposure estimation is in general performed for the general population and normal food consumers (eaters), by taking concentration and consumption data describing a central tendency (means and medians). To describe high consumers, EFSA (2008) has proposed to identify those foods that have the highest contribution to exposure and exchange the means by 95th percentiles in the estimation equation.

2. Ingestion of substances via the house dust and soil path

House dust and soil represent an important vehicle for nonvolatile substances. House dust consists of particles from several sources, e.g., soil dust, and from pollution. It contains a lot of different materials, e.g., plant pollen, mites, human and animal skin cells (dander), fibers, soil, and vapors. Substances migrate from the different materials (textiles, floor coverings, furniture, etc.) and, after release due to mechanical or thermic influence, adsorb to house dust. Partly, bigger particles may become a part of dust themselves.

The daily intake of house dust is unknown. Extrapolations from soil intake studies are normally used to estimate exposure from house dust intake. The intake of soil has been identified by means of tracer studies, taking substances that are poorly absorbed in the gut and comparing the concentrations measured in the stool with those in the soil. The AUH report (1995) recommends to take an estimate of 16 mg (median) and 110 mg (95th percentile) as standard values for house dust intake. The US EPA (2009) employs 60 mg per day as an estimate for central tendency. The extrapolation of soil to house dust may introduce uncertainties into the assessment; overestimation of exposure by house dust should be assumed. An actual overview about house dust exposure is given by Klenow (2019).

Exposure parameters needed to estimate oral exposure

- Concentrations of the substance in food and drinking water
- Consumption values for the food or drinking water, preferably related to individual body weight
- Weight
- Concentrations of substances in house dust/soil
- Default – values of house dust intake

Behavior of Populations and Individuals

Many exposed people are limiting their exposure by themselves and by their particular behavior. Studying the behavior in certain populations is essential and plays an increasing role in exposure assessment. The instructions of use will as well as the behavior of individuals and populations will influence the variability of the use and consumption of products. Two different types can be distinguished: (i) the active exposure where a person actively uses a product and (ii) the passive exposure where the exposed person is a bystander. In case of inhalation exposure, for example, the major difference between active and passive exposure is that the active person

may be closer to the source of exposure. An older version (3.0) of the ConsExpo tool is using a fictive room volume that is considerably smaller than the room to consider that situation. The indirect exposure via the environment is a particular form of passive exposure. From this perspective, eating food is passive exposure as well as being in a room and inhaling a substance that is released from furniture, while painting that furniture is active exposure.

Active and passive exposure can also be differentiated in terms of the degree of activity having impact for, e.g., exposure by inhalation. For example, the respiratory volume over time can vary from 15 m^3/day (at rest) up to 100 m^3/day (heavy work). This may lead to considerable variability in the exposure estimate and thus having impact for the risk characterization. When estimating exposure from inhalation, it is appropriate to assume a well-balanced ratio of activity and resting times.

Exposure Estimate

The aggregated (external) exposure is estimated from all external sources and via all pathways and routes of exposure.

If several agents are involved in the exposure scenario, the estimation is called cumulated exposure. Cumulated exposure is well characterized, for example, some pesticides or chlorinated dibenzo-dioxins, substance groups having some related toxicological features. Meek et al. (2011) also discuss combined exposures a situation where agents are involved having different toxic actions.

Time Budgets

As an important element of behavior scenarios, time budgets characterize the contact times of an exposed person. In case of exposure by inhalation, this is the time a person is staying in the room where the exposure takes place. Small children have normally longer contact times as adults because they may stay at home for longer time while adults are at work, outside, or at other business. This will change with school age. It is therefore of great importance to relate the time budgets to age. Data sources for time budget are, e.g., the US-EPA exposure factors handbook, the AUH (1995) report, and the RIVM general factsheet (Bremmer et al. 2006a).

Particular Age-Related Behaviors

The evaluation of behavior can be used to characterize important differences between adults and children. For example, the ingestion of soil and house dust may account for an important amount of oral exposure in small children. This occurs primarily in the toddlers, by crawling on the ground, as well as in the kindergarten, becoming less importance in the school age. Children frequently put their hand into the mouth, which is called the *mouthing behavior*. The latter has particular importance for exposure from

insecticides after treatment of pets against insects (lies, flies). Migrating substances from toys may also be relevant for mouthing. Therefore, migration rates are very important to estimate exposure. The mouthing time may vary over a big range (Groot et al. 1998; Juberg et al. 2001; Smith and Norris 2003). House dust evaluations represent an essential part of exposure assessment in children.

Exposure parameters needed to characterize a behavior scenario

- Duration of stay
- Frequency of staying
- Air ventilation
- Activities of "daily life"
- Exposure as active user or bystander
- Hand to mouth activities

Anthropometric Data

Exposure estimation needs anthropometric data that characterize the exposed person or population. Estimation of exposure by inhalation needs, according to the exposure scenario and the respective model, data about respiration rates and the lung surface. Dermal exposure evaluation requires information about body surfaces. However, estimation results are normally related to body weight. Relation to body surface is more appropriate, because body surface is correlating better with the extracellular fluid. Many substances distribute into body water, and there is also correlation between body surface and the basic metabolic rate. This is in particular of relevance when comparing results in children and adults.

Most important anthropometric data.

- Body surface and parts of body surface, e.g., hands and arms
- Body height
- Body weight
- Respiration time volume and related to activity
- Lung surface

Single-Point-Based (Deterministic) Exposure Assessments

Exposure factors can be characterized as single numerical values (deterministic approach) or as distributions (probabilistic approach). Therefore, every deterministic value represents a certain value from the distribution. An adequate exposure estimate must take into account all possible sources, pathways and routes which may result in very complex scenarios. The estimation is performed by separated estimations of the particular pathways with subsequent summation. Possible correlations of exposure paths must be considered. Also, summarizing exposure results should only be made for central tendency estimations. Results from individual conservative estimations, e.g., by using 95th percentiles or default values, should not be summarized. Consideration of conservative estimates must be performed very carefully, possibly by

addition of one conservative estimate with other averages. The European Food Safety Agency (EFSA 2008) has proposed to take the 95th percentiles of exposures contributing most to exposure, exchange them with the averages, and sum all up.

In many exposure calculations, arbitrary high values are used, in order to end up with an overestimation, without knowing the real situation. Such approaches are often lacking from reality.

Distribution-Based (Probabilistic) Exposure Assessment

When presenting exposure parameters as distributions, the bounds of that distribution may represent the entire estimation range. It is appropriate to use well characterized distributions and their statistical descriptors as a basis for exposure estimations. A check whether or not the used value can be matched with other representative values is needed. This approach will be facilitated considerably by use of modern computer tools. The total range and variability of the individual distributions will be weighted out and ends up with a distribution as result.

Probabilistic exposure modeling can be used as an alternative that considers the variability and uncertainty of the assessment that can be demonstrated by use of a probabilistic approach (Schümann 2019). Variability is characterizing the natural variation of parameters, while the uncertainty is determined by the lack of knowledge, which is often depending on data quality. For example, the body weight in the population participating in, e.g., the German food consumption study is described mostly by variability, because it is based on a representative sample from the entire population. On the other hand, the basis of data characterizing, e.g., concentrations of substances in products or food is often very poor. Therefore, these data must be considered uncertain.

Probabilistic models are formed by taking a similar general algorithm in the model but characterizing the model variables (parameters) as distributions. If the distributions are appropriately formed, i.e., the data basis is sufficient large and the values are representative for a population, the probabilistic distributions are describing the variability of the parameters. The less the number of data is and their representativeness, the more will distributions represent a mixture of variability and increasing degree of uncertainty.

Impact of Exposure Assessment

Exposure assessment represents, besides hazard identification, the second pillar that is needed for risk characterization. The margin between the quantitative estimate exposure and the N(L)OAEL) is characterizing the risk (risk characterization). It is called the margin of exposure (MOE), in earlier times the margin of safety (MOS), but both are meaning the same. The larger the MOS/MOE is, the more can the probability of risk be denied. A concern for risk is assumed if the exposure is exceeding the NOAEL. Risk can also be expressed as a ratio of the exposure dose

and the NOAEL (see resp. chapters in this book). Uncertainty factors are used in this formula to consider uncertainties, e.g., the lack of knowledge of the intraindividual and interindividual variation between animals and humans. Using the approach of tolerable/acceptable daily dose (TDI/ADI) or the acute reference dose (ARfD) the ratio should be lower than 1.

In the REACH regulation, the DNEL will be used instead of the NOAEL (compare the resp. chapter).

For these reasons it is of great importance to estimate the exposure as exact as possible. Estimates taking exposure scenarios and models are having sometimes considerable uncertainties, leading to partly extreme ranges of the exposure estimates which depend on the exactness of the description of the exposure scenario. It is essential to describe the exposure factors as exactly as possible. The approach of using conservative scenarios may lead to overestimations, resulting from rough models or taking defaults or other conservative values as model parameters. Due to the precaution principle, there is an intention to overestimate the exposure; it should, however, not result in unrealistic results. Distribution-based (probabilistic) modeling can be taken as an appropriate alternative because it considers the range of exposure factors and reveals a distribution of exposure. Taking distributions allows to consider extremes that characterize the skewness of a distribution. Ninety-fifth and higher percentiles are therefore appropriate descriptors of conservative assumptions and estimates and thus reflect "reality."

Measurements can be taken into account for exposure estimations, if they are representative for the population of interest. On the other hand, they are showing a *shot* of a particular event or situation which can hardly be transferred to a general scenario. Measurements available for, e.g., contaminants in food, in house dust, and indoor air should therefore be given attention, but they are not necessarily representative for the scenario of interest. Although there is a lot of data available for some substances, they often lack from representability and thus can be used for risk assessment only with great caution.

Cross-References

- ▶ Assessment of Background Exposure and Additional Exposure by Human Biomonitoring
- ▶ Exposure Analysis for Indoor Contaminants
- ▶ Importance of Exposure Level for Toxicological Risk Assessment
- ▶ Uncertainty Analysis in Exposure Assessment-Relevance for Toxicological Risk Assessment

Notes

1. The term "exposure parameters" summarizes the terms "exposure factors" and "exposure data" (Heinemeyer 2019)

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Quality Criteria for Primary Literature in Toxicology

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Klaus Schneider, Sebastian Hoffmann, and Agnieszka Kinsner-Ovaskainen

Contents

Abstract

Toxicological evaluations are based on the information derived from scientific studies. Understanding the quality of data is crucial for that purpose. Definition of evaluation criteria allows for a transparent and harmonized examination of the quality of toxicological studies.

Keywords

Toxicological studies · Study quality · Study reliability · Transparency · Evaluation criteria

K. Schneider (✉)
Forschungs- und Beratungsinstitut Gefahrstoffe GmbH, Freiburg, Germany
e-mail: klaus.schneider@fobig.de

S. Hoffmann
seh consulting + services, Paderborn, Germany
e-mail: sebastian.hoffmann@seh-cs.com

A. Kinsner-Ovaskainen
Institute for Health and Consumer Protection, European Commission DG Joint Research Centre, Varese, Ispra, Italy
e-mail: Agnieszka.KINSNER-OVASKAINEN@ec.europa.eu

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Introduction

Using toxicological studies of high quality and reliability for toxicological risk assessment is a prerequisite for reliable and trustworthy results of the assessment process. Current approaches for evaluating the quality and reliability of toxicological studies are presented.

Data and Data Sources

The OECD Test Guidelines and European Council Regulation (EC) 440/2008 for the testing of chemicals (see **Resources**) provide standardized, internationally agreed test method procedures used by government, industry, and independent laboratories to determine the safety/toxicity of chemicals and chemical mixtures, including pesticides and industrial chemicals. Standards of *Good Laboratory Practice* (GLP) set up rules for an adequate conduct of chemical nonclinical safety tests to ensure uniformity, consistency, reliability, reproducibility and contribute to the quality of the experimental procedures and data reporting (Borgert et al. 2016).

However, despite these standardization efforts, a large number of toxicological studies that can be found in the published literature have not been performed according to recent guidelines and/or GLP standards. This holds especially true for older studies and for scientific investigations, often conducted by academic research groups, aimed at answering specific questions.

Studies published in scientific journals (*primary literature*) are one of the main sources of information on toxicological properties of chemical compounds. The peer-review process should in principle implicate high quality standards of the published data. Depending on the regulatory area, nonpublished data from industry study reports constitute another important source of information for toxicological evaluations. Recent industry studies are generally performed according to the current guidelines and under GLP conditions.

Secondary literature (scientific reviews, or evaluations and reports from authorities or scientific organizations, which are increasingly available via internet portals such as eChemPortal, see **Resources**) allow for a quick data overview and may help identifying relevant studies. Nevertheless, the final evaluation of a certain substance should be performed on the basis of primary literature and original studies.

Evaluation of the Reliability of Studies

Comprehensive reporting of methodological details and results is a prerequisite for the evaluation of the reliability and thus quality of any toxicological study (Przybylak et al. 2012). Klimisch et al. (1997) proposed a system for categorizing the quality of experimental toxicological and ecotoxicological studies, which is now widely used in several regulatory schemes (e.g., the EU's REACH Regulation). In this system, studies are assigned one of the four categories as presented in Table 1.

Table 1 Klimisch categories for assessing reliability of toxicological studies

Klimisch category	Explanation
1	Reliable without restriction
2	Reliable with restriction
3	Not reliable
4	Not assignable

In order to conclude on a specific reliability category of published in vivo studies, the following criteria concerning the documentation of methodology and presentation of data should be assessed (summarized from Klimisch et al. 1997):

- Information on experimental animals (species, strain, gender, numbers, age)
- Information on the test substance (identity, purity, composition, source)
- Information on route of administration, dosage, and test conditions (e.g., methods for analytical verification of test concentrations)
- Information on performed examinations (endpoints investigated) and description of methods used (including statistical analysis)
- Description of observed effects and lesions
- Corresponding data of control group or historical controls of the laboratory
- Description of dose-response relationship, if applicable.

Nevertheless, the assessment of study reliability is often not straightforward, for example, when effects sizes are small, and may be influenced by subjective views and the experience of the assessor and biased by circumstances. In recent years, the issue of study quality has gained in interest, not at least triggered by the call for evidence-based approaches, such as systematic reviews (Stephens et al. 2016). Samuel et al. (2016) published an comprehensive review on existing guidance for assessing the methodological and reporting quality of toxicological data of various kinds (in vivo, in vitro, structure-activity relationships, physico-chemical, and human observational studies).

In order to support a harmonized and transparent evaluation of reliability of published in vivo and in vitro toxicological studies, a tool ("ToxRTool," see **Resources**) has been developed, which provides more detailed criteria for assigning Klimisch categories, and a way for transparent documentation of this evaluation (Schneider et al. 2009). Since its publication, ToxRTool was used for assessing the quality of toxicological studies for various purposes and topics, especially in the context of performing systematic reviews (e.g., Fewtrell et al. 2017; Nagy et al. 2020).

Science in Risk Assessment and Policy (SciRAP) is another data evaluation tool for toxicological and ecotoxicological studies. SciRAP (see **Resources**) was developed by Molander et al. (2015) and, rather than using a scoring system as in the ToxRTool, the evaluation relies on qualitative responses to the evaluation criteria, visualized by a color system. Recently, SciRAP was further evaluated by external experts and refined (Beronius et al. 2018).

With the advent of omics techniques new types of toxicological data are produced, for which the concept of quality assessment needs to be expanded. Vachon et al. (2018) developed a set of key criteria for such kind of data.

Plausibility of Study Design and Results

Besides reliability, further considerations are necessary to judge on the suitability of a study to be used in a substance safety evaluation. The comprehensive reporting of experimental designs and procedures for data evaluation allows for the examination of the consistency of the observations in relation to following aspects:

- Is the chosen design of the study (e.g., animal strain, cell line, route of administration, methods for statistical evaluation, etc.) appropriate for the question to be answered?
- Are the study results mechanistically plausible?
- Is the interpretation presented by the authors supported by the study results?

Plausibility of Results in Relation to the Overall Knowledge on a Given Substance

A final evaluation of a toxicological study should take into consideration how the presented results are related to the already existing knowledge on this substance. Contradictions between different studies have to be discussed taking into account possible explanations (e.g., differences in study design, animal strains, exposure route, etc.). Only then, a final conclusion on the adequacy of the study results for risk assessment purposes can be drawn. Often consideration of study reliability (as discussed here) is mixed with judging on the relevance or suitability of a study for a specific assessment purpose. Roth and Ciffroy (2016) emphasize the importance of keeping these two aspects separate.

Weighting of Borderline Cases

Toxicological evaluations and subsequent regulatory decisions on a given substance should be based on the entirety of available data, in accordance to the concept of *Evidence-Based Toxicology* (see **Resources**).

In cases where a toxicological study is of impaired quality, the weight or confidence attributed to this study should be determined in the context with other available evidence and the mechanistic plausibility. As an example: Should a study of restricted quality, which points out a much higher effect compared to similar studies be disregarded? This can only be decided on a case-by-case basis in the context the specific regulatory decision framework taking other evidence and potential explanations into account.

A *weight-of-evidence approach* can be used to integrate available information from various sources to draw conclusions. This weighting process requires various experiences, for example, in the evaluation of study quality, in the specific scientific domain, and in risk assessment in general. However, albeit being a quantitative method for combining evidence in support of a hypothesis, it is based on opinions that are influenced by individual expert's knowledge and personal backgrounds, which might lead to divergent decisions (Weed 2005). Therefore, the decision making process with respect to the study evaluation should be presented in a transparent, objective, and systematic manner.

Conclusions

Specified criteria for a formal evaluation of the reliability of toxicological studies have been developed and are well established. A comprehensive evaluation of quality of a primary source and its reliability for safety evaluation focuses on a consistent study design and the comprehensive documentation of methods and results. Furthermore, the internal plausibility of study design (e.g., appropriateness of the study design to address a specific question), as well as the plausibility of observations in view of existing information on the investigated substance should be evaluated to judge on the adequacy of study results for risk assessment. In equivocal cases, it is necessary to assess the weight of a specific study on a case-by-case basis, taking other evidence and potential explanations into account in a weight-of-evidence approach. This process of decision making has to be presented in a transparent and reproducible way.

Cross-References

- ▶ International Regulation of Toxicological Test Procedures
- ▶ Quality Assurance in Toxicology
- ▶ Toxicological Risk Assessment

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eChemPortal: http://www.echemportal.org/
Evidence-based toxicology: http://www.ebtox.com/
OECD Test Guidelines: https://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm
SciRAP: http://www.scirap.org/
ToxRTool: https://ec.europa.eu/jrc/en/scientific-tool/toxrtool-toxicological-data-reliability-assessment-tool

Data Mining in Toxicology

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Petra Wiedemeier and Inge Mangelsdorf

Contents

Abstract

Literature searches are necessary to find answers to many toxicological issues. Fortunately, today we are no longer reliant on time-consuming searches in reference books, but can make use of the Internet as an important tool for gathering information. A lot of information including complete substance assessments is easily available and free of charge. Because of the large variety of possible data sources, however, literature searches are difficult to undertake and in addition can take a lot of time. Depending on the particular issue of research, different searching strategies should be used.

P. Wiedemeier (✉) · I. Mangelsdorf
Department Chemical Safety and Toxicology, Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany
e-mail: petra.wiedemeier@item.fraunhofer.de

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Keywords

Bibliographical databases · Fact databases · Data search · Search methods · Search tools

Introduction

In the early 1960s, information on exposures to toxicants and toxins and their adverse effects was still restricted. Those who evaluated such information had to look it up in printed journals and reference books. At the same time, the number of synthetic chemicals increased continuously and there was a need for faster and sorted information for many professions including poison centers, forensic experts, food toxicologists, drug developers, and chemical safety experts in general.

Rapid advances in toxicological knowledge in the 1970s and 1980s were possible due to novel in vitro test systems (Ames et al. 1975; Greim et al. 1986), sensitive detection methods in histopathology and chemical analysis, and related developments. The first generation of small scientific calculators for laboratory use became available and personal computers were introduced. Thus it was possible for researchers to systematically collect toxicological information and evaluate laboratory data with statistical methods. This was also the beginning of databases and search systems used in toxicology (Kazyak 1974; Oxman et al. 1976; McGill et al. 1978). These became widely accessible after 1990 (Ludl et al. 1996; Gardner and Spangler 2000; Wexler 2001; Wukovitz 2001; Brinkhuis 2001).

Today huge databases and information systems are available for toxicology in general (see Wexler et al. 2020) and for subtopics like toxicological test guidelines (OECD 2020; ICH 2020) or glossaries like those of IUPAC (International Union of Pure and Applied Chemistry, see Duffus et al. 2017; Templeton et al. 2020). These together provide rapid access to the desired information and constitute an important tool for toxicologists and those involved in toxicological regulation.

Overview of Data Search Methods

Figure 1 gives an overview of different searching methods. The individual steps are described as follows.

Using Online Search Engines

The easiest way of performing a data search is searching in the Internet. Proven search engines include Google, Bing, Yahoo, and Google Scholar. If adequate search terms are entered, relevant Internet sites can often be found.

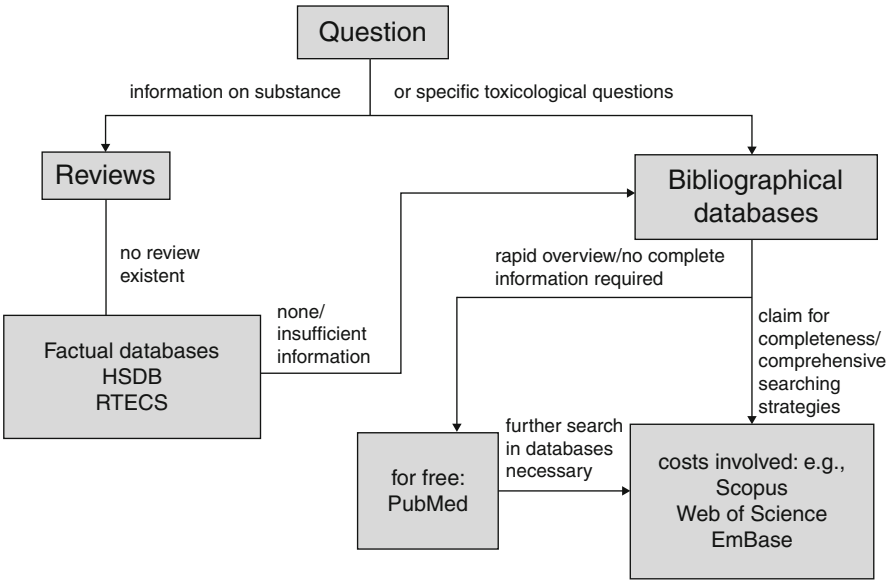

Fig. 1 Overview of possible data searching methods (example)

Portals of Publishing Companies

Meanwhile many publishing companies offer their journals and books online. As a result they provide the possibility to systematically search for specific literature on their homepages. Good online search tools for journals and e-books are offered, for example, by SpringerLink, ScienceDirect, and Wiley Online Library. For the download of articles a fee has to be paid in most cases.

Assessment by a National or International Committee

If the assessment of a particular substance is of interest, reports by expert committees can be helpful. The problem is that there exists no up-to-date collection of all reports available. The most comprehensive collection is offered by the OECD eChemPortal. It includes, for example, information and reports from the search portal INCHEM of the International Programme on Chemical Safety (IPCS) but also reports of the World Health Organization (WHO), the International Agency for Research on Cancer (IARC), and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Furthermore it provides access to reports of the Unites States Environmental Protection Agency (US-EPA), datasets of the International Uniform Chemical Information Database (IUCLID), and the registration dossiers submitted to the European Chemicals Agency (ECHA) under the European program for reevaluation of existing chemicals (REACH).

Reports of the United States National Toxicology Program (NTP) and the US Agency for Toxic Substances and Disease Registry (ATSDR) can be searched via the respective homepages of these organizations. Reports on chemicals that are consumer-relevant (e.g., fragrances, preservatives) can be found via the homepage of the Scientific Committee on Consumer Products (SCCP) of the European Union. Other important sources of information are documents justifying occupational exposure limits. Besides the German documents in this regard (MAK), also those of the English Health and Safety Executive (HSE), the Dutch Expert Committee on Occupational Standards (DECOS), the US Occupational Safety and Health Administration (OSHA), American Conference of Industrial Hygienists (ACGIH), and National Institute for Occupational Safety and Health (NIOSH) as well as the European Scientific Committee on Occupational Exposure Limits (SCOEL) and the Nordic Expert Group (NEG) are worth mentioning.

Review Articles

Furthermore, there are journals publishing exclusively review articles, such as Critical Reviews in Toxicology. These articles will be found by searching through the relevant bibliographical databases (see below).

Factual Databases

A good overview can also be obtained by querying fact databases. As the name indicates, these databases—in contrast to bibliographic databases (see below)—contain the relevant information about a substance and also provide references to the literature that was used as source of this information. Meanwhile the NLM (National Library of Medicine) has merged databases that have been established over years to existing databases. The best example for this is TOXNET which contains Toxline, HSDB, and CCRIS among others. Toxline has been completely absorbed into PubMed. HSDB (collection of referenced data derived from a core set of books, reports, and other literature) and CCRIS (carcinogenicity data from individual case studies) can be searched via PubChem. Access is still free of charge. Another frequently used factual database is RTECS, whose current data sets can be accessed free of charge via the homepage of the NIOSH Pocket Guide to Chemical Hazards.

Comprehensive Data Searches and Analyses

The search becomes a lot more complicated, if no assessments by expert committees and no review articles are available, or if preparation of such an assessment is actually the reason for the search. In this case, a comprehensive search in bibliographical databases becomes necessary.

Bibliographical Databases

Bibliographical databases, as the name indicates, provide the bibliographic information by which an article published in a journal (*primary literature*) or book can be found. In addition, most of these databases also include the abstracts of the publications.

Searches in bibliographical databases will yield journal articles but also book chapters, doctoral theses, and reports by research institutions. In toxicology in particular it is important to find also the so-called grey literature, meaning papers that have not been published in journals.

Selection of the Database

Important databases include MEDLINE, EMBASE, Chemical Abstracts (CA), BIOSIS, Web of Science, and Scopus. Furthermore, there are a few smaller databases tailored to specific subject areas. The databases differ in their thematic orientation. MEDLINE and EMBASE, for example, are focused on medicine, BIOSIS on biology, CA on chemistry. But there are also geographical differences. For example, European publications are taken into consideration much more in EMBASE than in MEDLINE.

For some years already, free access to MEDLINE (PubMed) has been offered in the Internet via the US National Library of Medicine (NLM). As the search and query options are limited there, they are not suitable for complicated issues. However, these databases can also be queried at a charge (e.g., via the providers DIMDI and STN). The search and query options are much better there but access is limited to trained staff and with costs.

For a very thorough and comprehensive search, several databases, therefore, have to be queried. But the problem is that most articles will be found more than once. Table 1 shows that, for the example of 2-butoxyethanol (CAS: 111-76-2) 645, citations were found in the SCOPUS database and that 216 of these will also be found, for example, in the PubMed database.

This is due to the fact that the different databases partly access the same publications. The major commercial database providers, therefore, offer the possibility to eliminate duplicates across different databases. Because of the more professional searching possibilities and the possibility to eliminate duplicates, it is

Table 1 Number of hits for the substance 2-butoyethanol in the databases PubMed, Web of Science and Scopus, and number of overlaps

	PubMed	WoS	Scopus
PubMed	**282**	204	216
WoS	204	**720**	444
Scopus	216	444	**645**

971 hits overall, 188 hits common in the 3 databases

recommendable to make use of an online database provider for comprehensive searches. The cost of a complex search, however, may then easily amount to several thousand euros. Alternatively, you can perform your own check for duplicates using the so-called reference management systems (see below). Citavi, EndNote, or Zotero are only a few, and there are many others available.

The search results depend on the substance being searched for. For poorly studied substances it is possible that not a single citation is found, while for well-studied substances such as polychlorinated biphenyls, there may be well over 30,000 hits in the available literature depending on the database.

Searching Strategies

Searches in factual databases are easy to perform, as they address a particular substance. Searches in bibliographical databases are more complicated, and you have to distinguish between a search for a particular substance name and a search for certain toxicological effects in this case. A chemical substance can be queried preferably via its CAS number, which unambiguously identifies the substance, alternatively by the international nonproprietary name (INN), chemical name according to IUPAC nomenclature, or the InChI identifier. Even CAS numbers cannot be queried in all databases. A small number of additional documents can be found by using synonyms, but many databases already include synonyms automatically. On the other hand, this may also result in related substances being found that are not actually being queried.

A search for toxicological effects is performed using specific search terms. Some databases (e.g., MEDLINE) offer structured lists of search terms (thesauri) that make it possible to search also for superordinate and subordinate terms. The database provider DIMDI offers a very helpful tool in this context, in the form of comprehensive search term lists in "preprocessed searches" (pps). Searches of this kind, however, often return a multitude of hits that are not really useful. The general rule is as follows: The more specific the search terms, the more appropriate the identified articles, but also the greater the risk of missing important publications. This means that the sensitivity of a search is adversely affected by high specificity and vice versa.

Reference Management

Helpful tools for managing large amounts of literature are commercially or non-commercially available software tools such as "EndNote," "Citavi," "Zotero," or "Faust." Most of these programs store not only the bibliographic information but also abstracts and key words. They allow systematic searches for specific search terms in fields like title, authors, year, journal, and more. Reference lists can be automatically generated in different formats. The access to individual databases and import of searched/found data into reference management systems is state of the art

now. This saves a lot of writing. However, the quality of the data is not the same with all databases, and if data have been imported from different databases, some manual post-editing is necessary in most cases.

Databases and Providers

A selection of relevant databases and database providers follows:

(Last visit to the websites: 2020-23-12)

Databases and Portals

Databases
CCRIS via PubChem: https://pubchem.ncbi.nlm.nih.gov/source/22070
HSDB via PubChem: https://pubchem.ncbi.nlm.nih.gov/source/11933
Medline via PubMed: https://pubmed.ncbi.nlm.nih.gov/
PubChem: https://pubchem.ncbi.nlm.nih.gov/
RTECS: https://www.cdc.gov/niosh/npg/npgdrtec.html

Database Portals
IPCS INCHEM: http://www.inchem.org/#/search
OECD eChemPortal: https://www.echemportal.org/echemportal/

Database Portals (Available at a Charge)
DIALOG: https://dialog.com/
DIMDI: https://www.dimdi.de/dynamic/en/homepage/index.html
STN: http://www.stn-international.de/

Databases (Available at a Charge)
Scifinder (CAplus): https://www.cas.org/products/scifinder
Scopus: https://www.scopus.com/home.uri
Web of Science: http://wokinfo.com/

Portals of Publishing Companies
Elsevier (ScienceDirect): https://www.sciencedirect.com/
Springer (SpringerLink): https://link.springer.com/
Wiley (Online Library): https://onlinelibrary.wiley.com/

Organizations
ATSDR: https://www.atsdr.cdc.gov/toxprofiledocs/index.html
ECHA, REACH: https://echa.europa.eu/
IUCLID: https://iuclid6.echa.europa.eu/
IUPAC: https://iupac.org/

JECFA: https://apps.who.int/food-additives-contaminants-jecfa-database/Search.aspx
NTP: https://ntp.niehs.nih.gov/publications/reports/index.html?type=Technical+Report
SCCP: https://ec.europa.eu/health/scientific_committees/consumer_safety/sccnfp_en
SCCS: https://ec.europa.eu/health/scientific_committees/consumer_safety/opinions_en
US-EPA: https://www.epa.gov/
WHO: http://origin.who.int/ipcs/en/ and/or https://publications.iarc.fr/

Statements Explaining Occupational Exposure Limits

ACIGH: https://www.acgih.org/
DECOS: https://www.healthcouncil.nl/about-us/the-council/permanent-committees/dutch-expert-committee-on-occupational-safety-decos
HSE: https://www.hse.gov.uk/
MAK: https://onlinelibrary.wiley.com/doi/book/10.1002/3527600418 and/or https://series.publisso.de/pgseries/overview/mak
NEG: https://www.av.se/en/the-nordic-expert-group/
NIOSH: https://www.cdc.gov/niosh/
OSHA: https://www.osha.gov/
SCOEL: https://ec.europa.eu/social/main.jsp?catId=148&langId=en&intPageId=684

There exist many additional systems for subfields of toxicology, such as QSAR (quantitative structure activity relationship), genotoxicity, or chemical substances (e.g., structures, properties, and alternative chemical names). The entirety of the sources provide the basis for solid information in regulatory toxicology.

Cross-References

► Checklist: Toxicological Risk Assessment in Practice
► Quality Criteria for Primary Literature in Toxicology
► Toxicology Report

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Principles of Analytical Chemistry for Toxicology

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Jürgen Durner and David C. Watts

Contents

J. Durner (✉)
Department of Conservative Dentistry and Periodontology, University Hospital, Ludwig-Maximilians-University of Munich (LMU), Munich, Germany
e-mail: juergen.durner@med.uni-muenchen.de

D. C. Watts
School of Medical Sciences and Photon Science Institute, University of Manchester, Manchester, UK

Otto Schott Institute of Materials Research, Friedrich-Schiller-University, Jena, Germany
e-mail: david.watts@manchester.ac.uk

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Abstract

In the first section, a historical summary of analytical chemistry is presented. In ancient history, one function of an analytical chemist was to confirm the identity of noble metals, especially gold. In later times, important inventions and their discoverer were named. Now, in the twenty-first century, analytical chemistry is an interdisciplinary scientific field.

Next, the aim and means of analytical chemistry are discussed. For analytical tasks, the chemist has available over 6,000 experimental procedures (including sub-specifications). The most important procedures are summarized. Moreover analytical problems, such as analyte(s) from complex matrixes, and the necessary purification as well as determination steps are discussed. Quantification measures, such as parts per trillion, are considered. The three analytical phases (pre-analysis, analysis, post-analysis) are presented, and recently developed analytical procedures such as "lab on a chip" and the "omics" sciences are introduced.

In the section "Pre-analysis," different techniques of sample preparation prior to analytical measurement are described. Apart from classic methods, such as crushing and homogenization, extraction techniques such as solid-phase extraction, liquid-liquid extraction and solid-phase micro-extraction are reviewed.

The analytical section is divided into three parts, plus subparts: (i) separation techniques are presented followed by (ii) atomic spectroscopy and (iii) selective analytical chemistry. Each (sub)part begins with a short historical overview. For separation techniques, first the principles of chromatography are described followed by the principles of electrophoresis and capillary electrophoresis. The chromatographic and atomic spectroscopy classifications and techniques are not presented in isolation, as in many analytical textbooks. They are described along with associated coupled techniques.

Such coupled techniques are liquid chromatography (LC), gas chromatography (GC), thin-layer chromatography, and ion exchange chromatography (IEC). LC is often coupled with mass spectrometry (MS; including different ionization techniques such as thermospray, fast atom bombardment, particle beam). GC is also often coupled with MS. Moreover derivatization techniques and headspace GC are presented.

In the case of atomic spectroscopy, atomic absorption spectroscopy (AAS) and inductively coupled plasma mass spectrometry (ICP-MS) are presented in more detail. In the section "Selective Analytical Chemistry," sensor techniques with ion-selective electrodes and the principles of immunoassays are described. These techniques are primarily for routine and fast analysis of known components in a sample. In most cases, the sample preparation steps are easy and rapid compared to, say, the sample preparation steps for gas chromatography.

Special types of mass spectrometers such as mass matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) and Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) and their usage will be described. These mass spectrometers can be coupled with LC.

Keywords

Analytical chemistry · History of analytics · Pre-analysis · Solid-phase extraction · Chromatography · High-performance liquid chromatography HPLC · Liquid chromatography-mass spectroscopy LC-MS · Gas chromatography-mass spectroscopy GC-MS · Headspace · Thin-layer chromatography · Electrophoresis · Atomic absorption spectroscopy AAS · Inductively coupled plasma mass spectrometry ICP-MS · Immunoassays · Matrix-assisted laser desorption/ionization time of flight MALDI-TOF · Fourier transform ion cyclotron resonance mass spectrometry FT-ICR-MS · Toxicology · Forensic medicine · Enzyme-linked immunosorbent assay ELISA

Introduction: Historical Summary

As an applied science, the history of analytical chemistry dates back to ancient history. Initially, analytical chemistry was a regulatory method to detect forgery of noble metals (Volke 2004). This was important because noble metals, especially gold, had an important function in the monetary system. In the early study of alchemy, which aimed to transform base metals to gold, analytical chemistry was necessary to check for any success.

Analytical chemistry is as old as chemistry itself because after any preparation steps, the result has to be verified. Normally, the desired molecule or compound was extracted, distilled, or precipitated from the reaction solution. In a further step, the separated component reacted with a second component to yield a substance that was then characterized by some distinctive physicochemical attribute. These could include its color, its melting or boiling point, its solubility in a series of solvent media, its smell, its optical activity, or its refractive index. Further quantitative analysis was achieved by gravimetric or titrimetric measurements. Many of the greatest discoveries in chemistry could fairly be described as classic examples of successful analyses, including the discovery of oxygen, the halogens, and several other elements. Discovering a new chemical element was regarded as the highest and most prestigious achievement possible for an academic chemist.

Parallel to the development of various chemical synthetic methods, special techniques were developed in the field of analytical chemistry. About 1660, R. Boyle (1627–1962) used litmus for the detection of acids and alkaline solutions. A. Lavoisier (1743–1794) investigated the composition of water (previously it was believed that water was an element) and published the law of conservation of mass. About 1800, J. Dalton (1766–1844) published his atomic theory and the law of multiple proportions (Dalton's law), and A. Avogadro (1776–1856) published his theory of gases. In 1817, J. Gay-Lussac (1778–1850) presented a volumetric procedure to determine the amount of silver in solution. He also accelerated the development of titrimetry.

In the nineteenth century, analysis became a recognized subdomain of chemistry. J. Berzelius (1779–1848) was one of its famous representatives (qualitative analysis,

law of definite proportions, chemical notation, discovery of elements. . .). In 1821/22, the German scientist C. H. Pfaff (1773–1852) published his two-volume book *Handbuch der analytischen Chemie für Chemiker, Staatsärzte, Apotheker, Oekonomen und Bergwerks Kundige* (Handbook of analytical chemistry for chemist, physician, pharmacist, economists and mining engineers (Pfaff 1821, 1822)). In 1861, R. Bunsen (1811–1899) and G. Kirchhoff (1824–1887) developed emission spectroscopy. In 1898, M. Curie (1867–1934) and her husband Pierre (1859–1906) discovered the elements polonium and radium. In 1894, the chemist W. Ostwald (1853–1932) published his book *Die wissenschaftlichen Grundlagen der analytischen Chemie* (The scientific basics of analytical chemistry (Ostwald 1894)). He explained many phenomena seen in analytical chemistry by the newly developing physical chemistry. Thus analytical chemistry has been jointly responsible for many central contributions to our understanding of nature (e.g., the existence of the various elements, gas theory, stoichiometry, atomic theory, the law of mass action, nuclear fission. . .).

In the twentieth century, with the knowledge transferred from other scientific areas, especially physics and engineering, new methods such as chromatography and spectroscopy were applied. About 1920, instrumental methods were introduced into analytical chemistry to support the classic methods of precipitation, extraction, and distillation. Several Nobel Prizes were awarded in the field of analytical chemistry, including W. Ostwald (1909; catalysis, chemical equilibria, and reaction velocities), F. Pregl (1923; quantitative organic microanalysis), A. Tiselius (1948; electrophoresis and adsorption in analytical chemistry, especially in the identification of blood serum proteins), A. Martin and R. Synge (1952; partition chromatography), J. Heyrovský (1959; polarography), R. Ernst (1991; Fourier transform nuclear magnetic resonance (NMR) spectroscopy), and J. Fenn and K. Tanaka (2002; both for their work in mass spectrometric analyses of biological macromolecules) as well as K. Wütherich (for his development of NMR spectroscopy for determining the three-dimensional structure of biological macromolecules in solution). Further aspects of the history of analytical chemistry are presented in the book by F. Szabadvary (Szabadvary 1966).

Analytical chemistry thus developed as an interdisciplinary scientific field spanning physics, biology, gene technology, toxicology, material sciences, engineering sciences, informatics, and (forensic) medicine.

The Aim and Means of Analytical Chemistry

C. R. Fresenius stated in his classic *Introduction to Qualitative Chemical Analysis* (Fresenius 1866):

> Chemical analysis is based directly on general chemistry, and it cannot be practiced without a knowledge thereof. At the same time it must be regarded as one of the fundamental pillars upon which the entire scientific edifice rests; for analysis is of almost equal importance with respect to all the branches of chemistry, the theoretical as well as the applied, and its

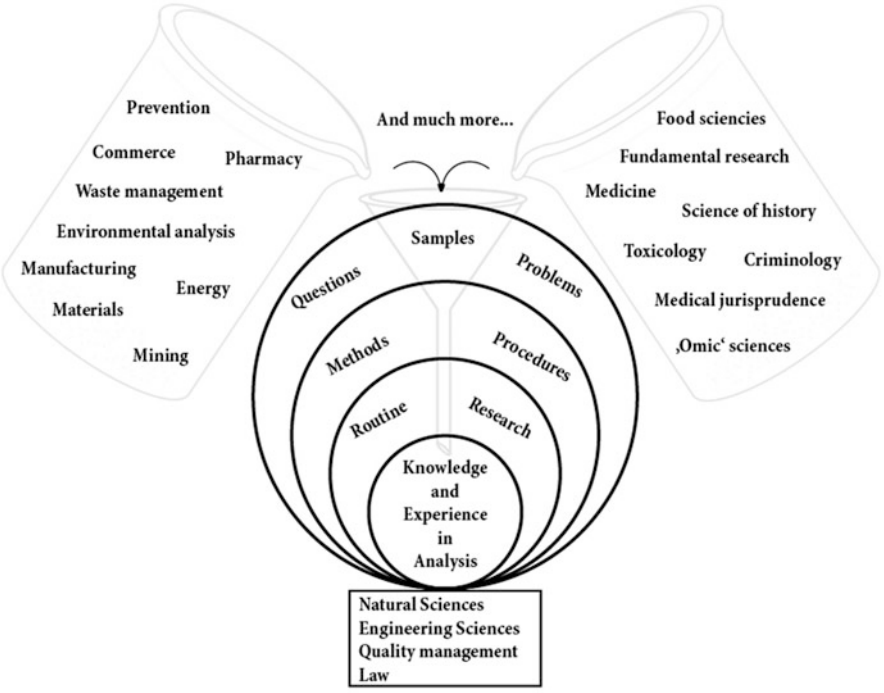

Fig. 1 Challenges for the analytical chemistry. Samples from all areas are brought to the analyzer. With his spectrum of methods and – last but not least – his experience and knowledge, he analyzes the samples. Moreover the analyst has to take into account current laws, quality management standards, and new technical developments in his field. (Modified after Tölg et al. (2000))

> usefulness to doctors, pharmacists, mineralogists, enlightened farmers, technologists, and others requires no discussion.

Analytical chemistry (often also called analysis – the word is a transcription of the ancient Greek αναλυσις meaning "resolution") penetrates all areas of live and working (Fig. 1).

It is normally divided into different subspecies, depending on the field of application, such as environmental analysis, biological analysis, geological sciences, online process analysis, food analysis, and instrumental analysis as well as forensic science and materials characterization. Analytical chemistry is not only concerned with trace analysis but also analysis of bulk substances or ingredients, as in the food industry.

In more general terms, analytical chemistry is concerned with methods to determine the chemical composition of different samples, including trace or bulk analysis. Basic issues in analytical chemistry are sensitivity, selectivity, and accuracy. The speed of determination is now especially important in clinical chemistry and forensic science. The analytical chemist is normally confronted with two questions: (1) What

substances or chemical groups are present in the sample (*qualitative* chemical analysis, molecular structure analysis)? and (2) What is their content in the sample (*quantitative* chemical analysis)? In the most cases, it is unnecessary to know the full chemical composition of the sample but only the most important compounds of interest.

In practice, qualifying and quantifying an analyte in a complex sample becomes an exercise in problem-solving. The sample can be regarded as the sum of two parts: the *matrix* and the *analyte(s)*. To be efficient and effective, an analytical chemist must know the appropriate tools to tackle a wide variety of problems. Therefore analytical chemistry requires a broad education in chemical and physical concepts. Advanced separation and spectroscopic techniques, as well as data analysis ("chemometrics"), play an important role in this field.

For the analytical task, over 6,000 experimental procedures including sub-specifications are available. A selection of the most important methods are listed in Table 1 (Durner 2010).

To isolate the analyte from a complex matrix, two further questions arise for the chemical analyst in practice:

1. Which steps of purification and isolation are necessary for the determination (qualitative and/or quantitative) of the analyte(s)?
2. Which method of determination is suitable for my analyte(s)?

To accomplish these goals, a sample is prepared by traditional methods like dissolution, homogenization, extraction, filtration, evaporation, separation, chemical derivatization, as well as newer methods like solid-phase extraction. Traditional and new methods are often combined to reduce the number of (time consuming) preparation steps; and the degree of automation is increasing. The achievements of analytical chemistry, especially in inorganic or organic trace analysis, can be shown by the amounts that can now be detected. To emphasize what **ppm** (parts per million), **ppb** (parts per billion), **ppt** (parts per trillion), or **ppq** (part per quadrillion) mean, we can use mass or volume units:

ppm is comparable to mg/kg, µg/g or mL/m^3, µl/L.
ppb means µg/kg, pg/g or $\mu l/m^3$, nL/L.
ppt is the same as ng/kg, pg/g or nL/m^3, nL/L.
ppq means pg/kg, fg/g or pL/m^3, fL/L.

For such trace analysis, as well as for normal analysis, quality management plays a very important role. To guarantee cross-border equivalence in the field of analytical chemistry, international rules for laboratory working such as "good laboratory practice (GLP)" or European and International Standards like EN ISO 17025 were introduced.

In analytical chemistry, three phases can be distinguished.

Table 1 Selection of the most important methods in analytical chemistry (Durner 2010)

Spectrometry	
Absorption spectroscopy/photometry	Nephelometry/immunonephelometry
UV-/VIS-/NIR-/IR-spectroscopy	Turbidimetry/immunoturbidimetry
Atomic absorption spectroscopy (AAS)	Atomic emission spectroscopy (AES)
Atomic fluorescence spectroscopy (AFS)	Flame emission spectroscopy
Nuclear magnetic resonance (NMR) spectroscopy	Inductively coupled plasma mass spectrometry (ICP-MS)
Luminescence spectroscopy: bioluminescence measurement, chemiluminescence, fluorescence, time-resolved fluorescence, fluorescence polarization, and phosphorescence spectroscopy	Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS)
Ligand assays	
Enzyme immunoassay	Fluorescence polarization enzyme immunoassay
Fluorescence immunoassay	Radioimmunoassay
Immunoblot (Western blot)	Receptor assay
Luminescence und electrochemiluminescence-immunoassay (CLIA/ECLIA)	
Chromatography	
Thin-layer chromatography (TLC)	Gas chromatography (GC) and GC-MS
Liquid chromatography (LC)	High-performance liquid chromatography (HPLC) and HPLC-MS
Electrophoresis	
Zone electrophoresis: cellulose acetate electrophoresis	Rocket electrophoresis
Immunoeleetrophoresis/Immunofixation	Isotachophoresis
Isoelectric focusing	Capillary electrophoresis
Pulse-field gel electrophoresis	Counterimmune electrophoresis (countercurrent electrophoresis)
Blood cell counts	
Impedance measurement	Immunophenotyping of hematopoetic cells (flow cytometry)
Flow cytometric cell count determination with cytometric or cytochemical-cytometric cell classification particle property determination with automated processing (particle counting and particle size determination of blood cells)	
Electrochemical studies	
Amperometry: O_2 partial pressure (Clark electrode)	Coulometry
Potentiometry: pH, ion-selective electrodes	Voltammetry

(continued)

Table 1 (continued)

Molecular biological methods	
(Real-time) polymerase chain reaction (PCR)	Fluorescence in situ hybridization (FISH)
Southern blot	Evidence for single-nucleotide polymorphisms (SNPs) with, e.g., restriction fragment length polymorphisms (RFLPs), fluorescence resonance energy transfer (FRET)-probes, density gradient gel electrophoresis (DGGE), denaturing HPLC (DHPLC)
Additional procedures	
Aggregometry	Areometry
Filtration (adsorption filtration, membrane filtration, ultrafiltration)	X-ray diffraction
Immunohistochemistry	Coagulometry
Microscopy (light and dark field, fluorescence, and phase-contrast microscopy)	Osmometry: cryoscopy, vapor-pressure osmometry
Qualitative studies with visual evaluation (e.g., osmotic erythrocyte resistance)	Sedimentation studies (erythrocyte sedimentation rate)
Reflectrometry/carrier-bonded methods of analysis	Radioactivity measurement
Rheology, viscosimetry	Centrifugation: analytical ultracentrifugation, density-gradient centrifugation

1. Pre-analysis
2. Analysis
3. Post-analysis

The term “pre-analysis” or “pre-analytical phase” encompasses all the administrative and functional factors and processes that occur prior to laboratory analysis. These include preparation, isolation, work-up by centrifugation, storage, and transport. The term “analysis” or “analytical phase” covers taking aliquots and the general preparation of an analytical sample, the analysis itself, and acquisition of the appropriate data value(s). In this phase precision, accuracy, detection limit, method specificity, analytical sensitivity, and statistical quality control play an important role. The term “post-analysis” or “post-analytical phase” covers the analytical assessment of analytical results and the recorded set of definitive findings. Keywords in this context are plausibility, trend analysis, abnormal values, status assessment, diagnostic sensitivity, diagnostic specificity, and predicted value (O’Kane et al. 2008).

A few years ago, a new technology was introduced which has started to change classical laboratory chemistry in some areas. It uses “lab on a chip” devices that have the size of a credit card or even a fingernail. A “lab on a chip” allows determination of multiple analytes without further matrix purification. Fixed capture molecules on

the chip sensor array bind molecules, such as nucleic acids or blood proteins, from the fluid matrix (a few femtoliters of which are normally sufficient). The sensor array may incorporate electrochemical sensors that recognize the binding of a substance by the change in potential or current flow. As this chip technology is derived from the field of electronics, the chips are rather expensive. Therefore inexpensive alternatives, such as paper-based "chips," are in development (Yehya and Wael 2010; Trietsch et al. 2011; Deisingh and Thompson 2004; Palchetti and Mascini 2008).

This new micro-fluidic technology is important for medical analysis as well as for basic "omics" sciences (proteomics, metabolomics, etc.). This technology has been praised as offering "devices suitable for every purpose" to find the proverbial "needle in the haystack" (Schneider and Orchard 2011; Saleem and Reddy 2011; Singh et al. 2010; Suter-Dick and Singer 2008).

Important Techniques of Pre-analysis

For the (trace) analysis of different substances from complex matrices, special treatments are necessary to determine the analyte(s) with sensitive analytical methods (Peters and Remane 2012). This field of pre-analytics involves different techniques of sample taking and especially different possibilities of sample preparation prior to the analytical measurement (Persoon et al. 2006). Examples of pre-analytical methods, for "working up" a sample, are crushing, homogenization, solubilizing, chemical exploration, and extraction techniques. In toxicology, extraction techniques play an important role. Some long-established extraction techniques are based on the principle of two non-mixable phases in close contact (Hennion 2000). An overview of common extraction techniques is shown in Table 2.

Table 2 Overview of widely used extraction techniques. (Modified after Gey (2008))

Phase 1	Phase 2	Extraction technique
Solid	Liquid	Solvent extraction techniques, e.g., Soxhlet extraction, ultrasonic or microwave-assisted extraction, accelerated solvent extraction
Solid	Supercritical	Supercritical fluid extraction
Solid	Gas	Gas extraction techniques, e.g., headspace extraction
Fluid	Solid	Adsorptive extraction techniques like solid-phase extraction (SPE), solid-phase microextraction (SPME), or adsorptive microextraction techniques as well as dispersive extraction techniques
Liquid	Liquid	Liquid-liquid extraction techniques, e.g., liquid-phase extraction, liquid-phase microextraction (incl. hollow fiber techniques), ion pair extraction
Liquid	Gas	Purge and trap techniques (PT techniques)
Gas	Liquid	SPME
Gas	Solid	SPME

Solid-Phase Extraction

Solid-phase extraction (SPE) is a chromatographic technique known for over 60 years (Liska 2000). It is a physical extraction process to enrich, isolate, and/or cleanup the analyte(s) from a complex liquid matrix onto a stationary phase from the SPE material. This technique can be very effective, even when the solutes are present in extremely dilute concentrations (e.g., ppb). The extraction tube is usually packed with an appropriate bonded phase that is reproducible in activity, selectivity, and retention properties. In the first step, an adsorption on the solid phase takes place that means that the interaction of the analyte(s) and the solid state is stronger compared to the liquid phase. In the second step, an extraction from the solid phase takes place. In other words, the interaction of the analyte(s) and the liquid phase is now stronger compared to the solid phase. Therefore it is possible to retain and enrich the analyte (s) in the first step and to elute it in the second step by rejecting the matrix. Because it is widely used, the SPE technique is discussed in more detail. The principal setup of a SPE tube is shown in Fig. 2. The I.D. of a tube is in the range 2–10 mm, and it is 2–4 cm long and is usually made from an inert polymer or, occasionally, from stainless steel or other materials. Normally the first step in using SPE tubes is conditioning. This means that the solid phase (sometimes also called the adsorbent) is pretreated with an (organic) solvent. This is necessary to activate the side chains from the solid phase to get a high and reproducible recovery. After conditioning, the sample can be placed. With the help of a vacuum, the sample is drawn through the solid phase of the tube. Afterwards, the solid phase is washed. In the next step, the solid phase is dried and then the analyte(s) are eluted by a solvent or a series of solvents/solvent mixtures of successively increasing elution strength (an eluotropic series) (see Fig. 3).

Effective separation by SPE depends primarily on the proper choice of sorbent and eluting solvents depending on the chemical and physical properties of the components in the sample. SPE tubes are available incorporating a wide variety of chemistries, adsorbents, and sizes. The most commonly used phases for the solid state are reversed phase, normal phase, ion exchange, and adsorption (Camel 2003; Majors 2010; Zwir-Ferenc and Biziuk 2006).

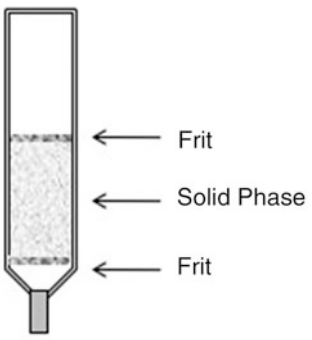

Fig. 2 General setup of a solid-phase extraction (SPE) tube

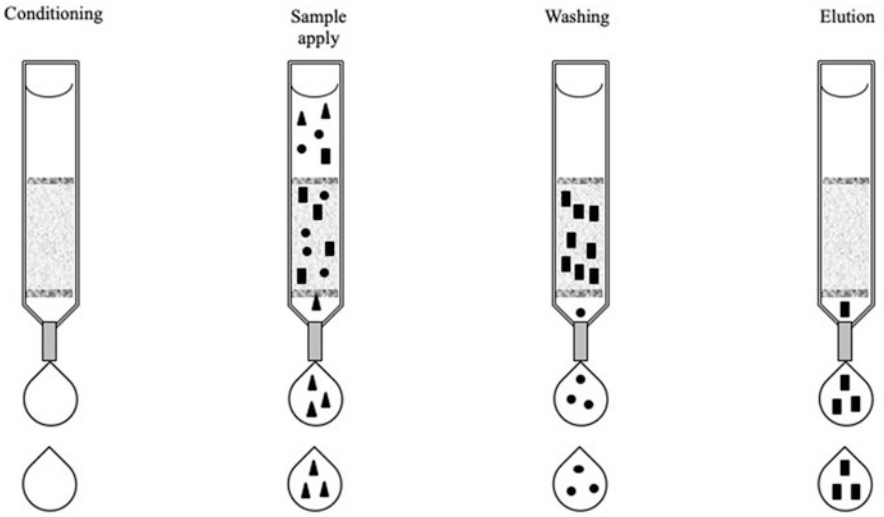

Fig. 3 Working principle and general steps in using solid-phase extraction (SPE) tubes. The first step is conditioning: i.e., the cleaning and activating of the solid phase of SPE. The second step is normally the sample application. Only the desired analyte(s) should be adsorbed, and these can be enriched. The third step is a washing step and in the fourth step the analyte(s) are eluted

Liquid-Liquid Extraction

Liquid-liquid extraction (LLE) is a tool for separating and isolating the favored analyte (s) of a liquid mixture by contacting it with a second, immiscible liquid in which one or more of the favored analyte(s) are preferentially soluble. Normally one of the two phases is an organic phase, while the other is an aqueous phase. Under equilibrium conditions, the distribution of analyte(s) over the two phases is determined by a distribution law. In practice, it is not always possible to find the optimum conditions that provide both high recovery and high purity of the analyte(s) in one extraction step. Therefore it is not unusual that a second extraction procedure, with a different solvent or other extraction conditions (e.g., pH value), is necessary. Moreover multiple extraction steps with the same solvent can also be required. In the case of a large extraction volume, the solvent must be evaporated to enrich the analyte(s) (Hii and Lee 2010; McConvey and Nancarrow 2011; Silvestre et al. 2009; Tedder 2009; Testard et al. 2010).

Solid-Phase Microextraction

Solid-phase microextraction (SPME) is a common, solventless, fast, and field-compatible technique for extraction and concentration of volatile and semi-volatile analyte(s). It was invented in 1990 by Dr. J. Pawliszyn and colleagues. The physical basics are adsorption and desorption of the analyte(s) from a polymer-coated fused fiber (Pawliszyn 1997). In SPME, analyte(s) establish equilibrium between the sample

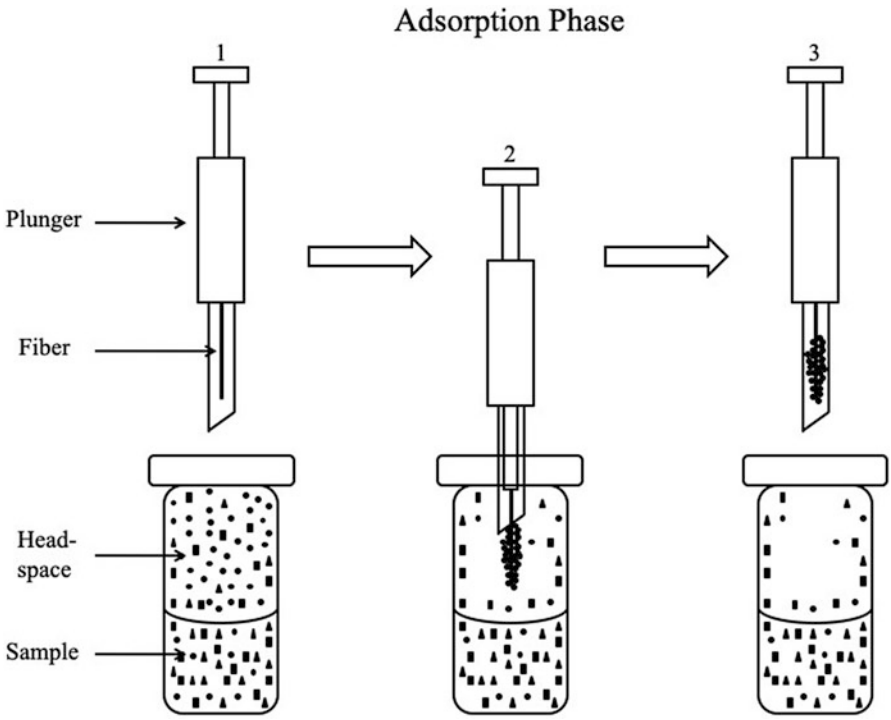

Fig. 4 **Adsorption phase**. First, the SPME needle pierces the septum on the sample container. Second, the SPME fiber is incubated in with the analyte(s). Third, after incubation from minutes to hours independent from, e.g., the concentration of the analyte(s), the SPME fiber is retracted and the needle is withdrawn

matrix, the headspace above the sample, and a polymer-coated fused fiber (SPME adsorptive layer; see Fig. 4). After the sampling period, during which extraction has ideally reached equilibrium, the adsorbed analyte(s) is/are transferred into an inlet system that desorbs the analyte(s) from the SPME adsorptive layer into a gas (for GC) or liquid (for LC) mobile phase (see Fig. 5). Because analyte(s) is/are concentrated on the SPME fiber, and is/are rapidly delivered to the column, minimum detection limits are improved and resolution is maintained. SPME provides linear responses for wide concentrations of analyte(s). By controlling the polarity and thickness of the coating on the fiber, maintaining consistent sampling time, and adjusting several other extraction parameters, an analyst can ensure highly consistent, quantifiable results from low concentrations of analyte(s) (Chen and Pawliszyn 2007). Sometimes a secondary trapping and release of desorbed solutes after SPME is necessary when desorption from the SPME adsorption layer is too slow. This trapping and release can be accomplished using a discrete thermal trap or, in the case of column stationary-phase trapping, by injection onto a cold column and subsequently temperature programming for solute elution (Duan et al. 2011; Risticevic et al. 2009; Risticevic et al. 2010; Vuckovic et al. 2010).

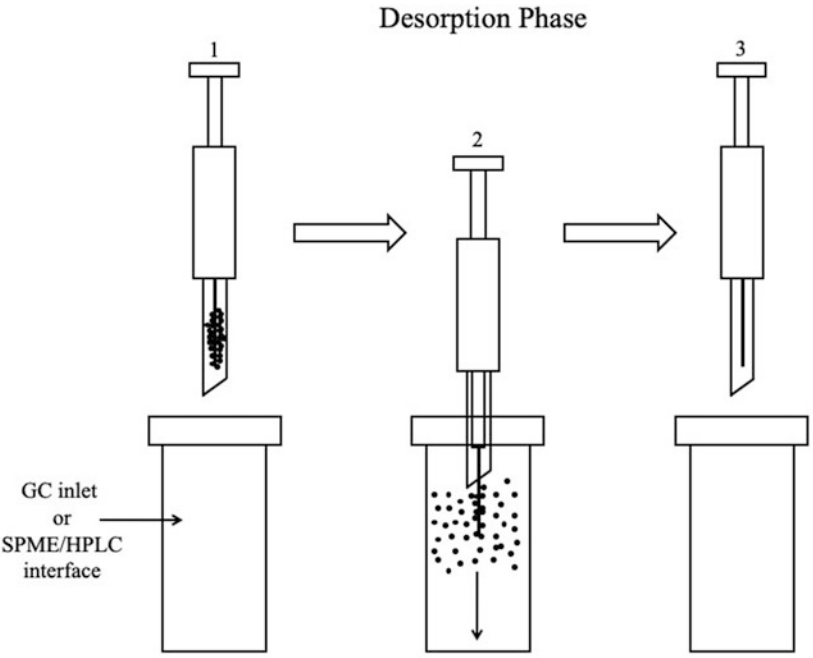

Fig. 5 **Desorption phase**. First, the SPME needle pierces the GC inlet or the needle is introduced into the SPME/HPLC interface. Second, the analyte(s) on the SPME fiber are desorbed, e.g., by heating the inlet. Third, the SPME fiber is retracted and the needle withdrawn

Separation Techniques

Chromatography

Chromatographic methods have been used for a long time. In ancient Greece, Aristotle used alumina for the cleaning of seawater. In 1859, the German scientist F. Runge made experiments that were a precursor to paper chromatography (Runge capillary pictures). In 1901, the Russian botanist M. Tswett initially described the method to separate plant pigments such as chlorophyll and carotenes (Furr 2004). Since 1906, the term "chromatography" was used, derived from the Greek words "χρωμος, chromos" "color" and "γραφειν, graphein" "to write," meaning "color writing."

The physical bases of chromatography are the chemical and/or physical interactions of the analytes from the sample, present in the mobile phase, with the particles of a stationary phase – resulting in a temporal and spatial separation of the analytes (retention of the analytes; see Fig. 6). The greater the affinity of the analyte to the stationary phase, the greater the delay period during chromatography. The separated analytes are detected at the exit of the column by a flow-through detector that

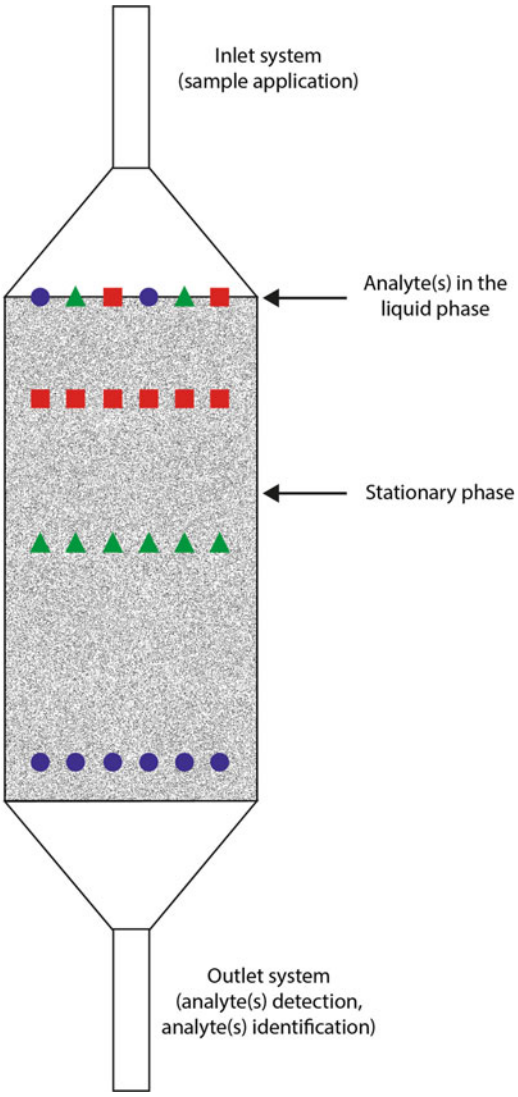

Fig. 6 Principles of chromatography. The sample with the analyte(s) is applied to the chromatographic system. The analyte(s) in the mobile phase interact with the stationary phase. Thereby the separation takes place. After leaving the chromatographic system, the analyte(s) can be detected by different analyzing systems

measures their quantity. The result of the separation is a chromatogram (see Fig. 7), where the signal intensity is shown as the ordinate and the retention time as the abscissa. The different retention times are characteristic for the substance. The height of the signal/peak, or the area under the signal/peak, can be used for quantification of the analytes' concentration (Guiochon and Trapp 2000).

One possible classification of chromatographic techniques considers the following points (see also Fig. 8):

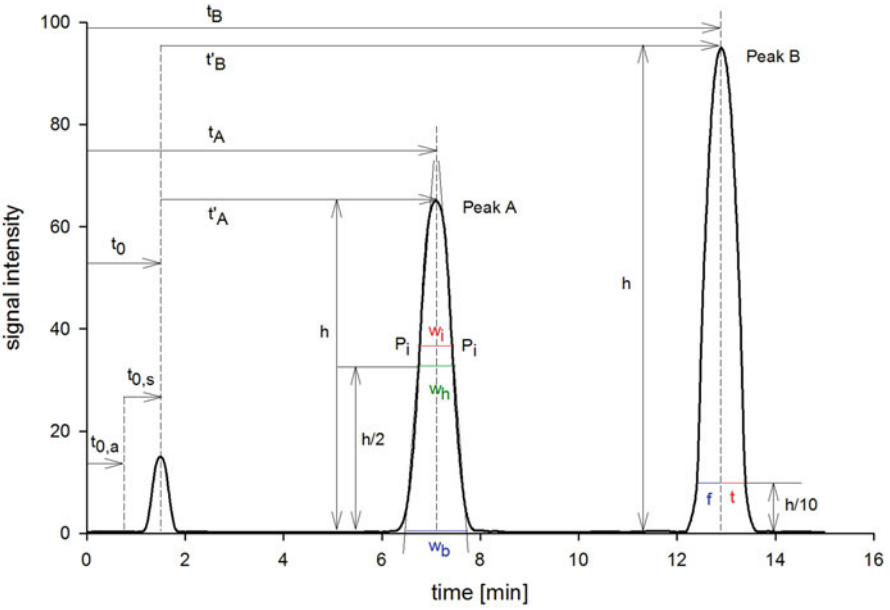

Fig. 7 **Example of a chromatogram $\mathbf{t_{0,a}}$**: Time delay of the apparatus. This is the time the eluent needs to reach the detection system from the injector. It should be as low as possible ($t_{0,\,a} \approx 0$) $\mathbf{t_{0,c}}$: Time delay of the column. This is the time the eluent needs to pass the column. $\mathbf{t_0}$**:** Dead time. This is the time the eluent needs to pass the distance from the injector through the column to the detector. $t_0 = t_{0,\,a} + t_{0,\,c} \approx t_{0,\,c}$ **t** Retention time of the components. This is the time required for the components to pass the distance from the injector through the column (including interaction) to the detector. **t'** Net retention time for a component. This is the time the component is in the stationary phase. $t' = t - t_0$ **k'** Retention factor (formerly called as capacity factor). k' is a characteristic parameter of a component in the phase system. $k' = \frac{t'}{t_{0,c}} = \frac{(t-t_0)}{t_{0,c}} \approx \frac{(t-t_0)}{t_0}$ **T** Tailing factor. This is a measure for the symmetry of a peak. It is measured at 10% peak height after the International Union of Pure and Applied Chemistry (IUPAC) or 5% peak height after the US Pharmacopeia (USP). $T = \frac{t}{f}$ **a** Separation factor (formerly called the selectivity). **a** characterizes the potential of a phase system to separate two components. In the numerator the data of the later eluted substances are named. Therefore **a** is ≥ 1. $\alpha = \frac{t'_B}{t'_A} = \frac{k'_B}{k'}$ **R** Resolution. Considering the width of a peak it characterizes the separation of two neighboring peaks. $\Delta t_{B,\,A} = t_b - t_a$; $R = 2 \cdot \frac{\Delta t_{B,A}}{w_{b,A}+w_{b,B}} = 1,177 \cdot \frac{\Delta t_{B,A}}{w_{h,A}+w_{h,B}}$

(a) Selection of the separation distance, as in column or layer chromatography
(b) Selection of the phases:

Normally, the mobile phase is liquid or gaseous, and the stationary phase is solid or liquid. So four combinations can result: liquid-solid and liquid-liquid as used in liquid chromatography (LC); gaseous-solid and gaseous-liquid as used in gas chromatography (GC).

(c) Selection of the separation mechanisms, such as separation by adsorption, distribution, ion exchange, and cavity diffusion (molecular sieve chromatography, gel permeation chromatography).

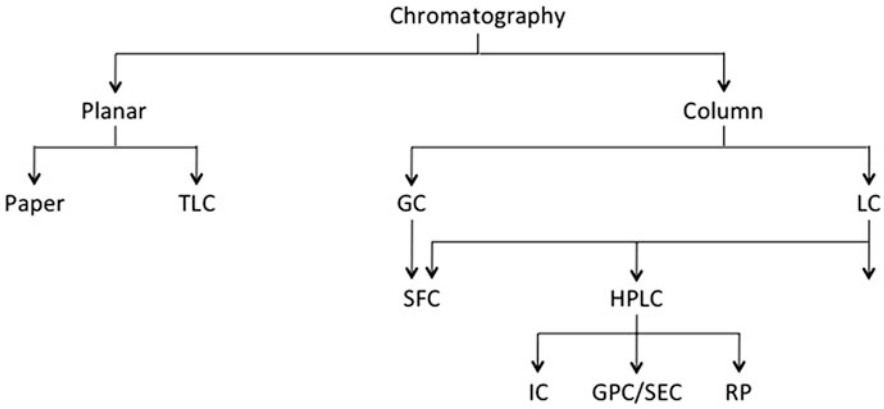

Fig. 8 One possible classification of chromatographic techniques. TLC = Thin-layer chromatography; GC = gas chromatography; LC = liquid chromatography; SFC = supercritical fluid chromatography; HPLC = high-performance liquid chromatography; IC = ion chromatography; GPC = gel permeation chromatography; SEC Size exclusion chromatography; RP = Reversed phase chromatography

In the following, important chromatographic techniques will be presented together with a widely used coupled technique: mass spectrometry.

HPLC: Including Coupled Techniques

High-performance liquid chromatography (HPLC; formerly also called high-*pressure* liquid chromatography) is a technique of liquid chromatography and a highly improved form of column chromatography. In comparison to traditionally chromatography, a solvent is not allowed to drip through the column under gravity; instead it is forced through under high pressure – up to 400 atmospheres. The HPLC instrument consists of a solvent reservoir, degasser, pump, injector, detector, integrator, and column, the last of which is a temperature-controlled oven. Advantages of HPLC techniques compared with traditional chromatography are:

- The analysis time is shorter.
- It is possible to use much smaller particles as the stationary phase in the column that gives a greater surface area for the interaction between the analytes and the stationary phase. In consequence, the separation of a multicomponent mixture is better, the reproducibility is higher and the detection limit is lower.

Depending upon the interaction between the particles of the stationary phase and the analytes in the liquid phase, the following kinds of liquid chromatography are distinguishable:

- Exclusion chromatography
- Ion chromatography

- Adsorption chromatography
- Chromatography of optical isomers.

Adsorption chromatography is widely used. Depending upon the stationary phase, "normal phase" and "reversed phase" chromatography can be distinguished. The stationary phase in normal phase chromatography is made of unmodified silica gel and in rare cases of Al_2O_3. Because of the polar character of the stationary phase, the components (eluents) of the mobile phase are nonpolar like hexane. Normal-phase chromatography is used in about 5–10% of routine measurements.

In reversed phase chromatography, the stationary phase is more nonpolar (hydrophobic) compared to the mobile phase ("reversed phase"). The free silanol groups of the silica gel have been reacted with alkyl chlorosilane to form siloxane groups. One common stationary phase uses the n-octadecylsilane (OSD) modification of the silica gel. For reversed phase chromatography, mixtures of water, methanol, tetrahydrofuran (THF), or acetonitrile (ACN) are used as mobile phases. The most polar analytes of the sample will be eluted first because their interaction with the hydrophobic groups of the stationary phase is weak, whereas the most nonpolar analytes will be eluted at longer retention times because their interaction with the stationary phase is greater (whereas, in the case of normal phase chromatography, the elution sequence is inverted).

Another important factor in chromatography is the temperature. It influences the interaction between mobile and stationary phases as well as the viscosity of the liquid phase. For high reproducibility and robustness, it is important to use a constant temperature (Meyer 2010).

The composition of the liquid phase (often also called the elution phase) can be either constant or varying. The first case is named *isocratic elution* and the second case *gradient elution*. The gradients can be linear, concave, convex, or in steps. Besides binary gradients also ternary or quaternary gradients can be used, involving three or four different elution media.

The correct flow rate of the liquid phase also depends on factors such as the internal diameter of the column. At high flow rates, the interaction of the analytes with the stationary phase is insufficient. It is advisable to choose a constant velocity (that a linear flow pattern results) when attempting to reproduce chromatography results obtained with columns of differing internal diameters.

In HPLC, the analytes can be detected using different detector types. The detectors are classified into two groups: The first group uses certain characteristics of the total elution flow (depending on the dissolved analytes therein) such as density, refractive index, capacitance. The second group uses certain characteristics of the dissolved analytes such as UV-absorption, fluorescence, redox behavior. The choice of the detector type depends on the analytes. In some cases, multiple detection systems are used. Typical detector types use the principles of UV/VIS, (FT)-IR, fluorescence, electrochemical, conductivity, refractive index detectors, evaporative light scattering detectors, and mass spectrometric detectors (LC-MS; see section "LC-MS (Thermospray, Fast Atom Bombardment, Particle Beam)").

Many substances of current interest cannot be detected by HPLC because they do not contain the necessary chromophoric, or fluorophoric, groups. In this case, it is possible to add a chromophoric group by a derivatization reaction (the derivatization process is discussed in section "GC: Including Coupled Techniques"). Derivatization can be done in a pre-column mode (that means before analytical separation) or in post-column mode (after separation). Typical chromophoric groups for UV/VIS detection are, e.g., 4-dimethylaminoazobenzene-4′-sulfonyl chloride, 1-fluoro-2,4-dinitro-benzene (Sanger's reagent), and 4-(dimethylamino)benzaldehyde (Ehrlich's reagent), and for fluorimetric detection fluorescamine, 4-(dimethylamino-sulfonyl)-7-fluoro-2,1,3-benzoxadiazole, and 3,4-dihydro-3,4-dioxo-1-naphthalenesulfonic acid sodium salt (Folin's reagent). HPLC derivatization plays an important role in the determination of many pharmaceutical compounds(amino acids, antibiotics), in agrochemistry (proteins, peptides, toxins), in the environment (pollutants), and in food sciences (biogenic amines as indicators of protolytical process) (Kaushal et al. 2011; Milroy et al. 2012; Gianotti et al. 2011; Polettini 2011).

This chapter will only describe selected methods with practical use in the field of toxicology. Therefore molecular spectroscopic techniques like mass spectrometry (MS), matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) will be presented as coupled technique with the chromatographic methods and not separately, as in textbooks of analytical chemistry. Atomic spectroscopic techniques that are widely used in the field of toxicology are considered in section "Atomic Spectroscopy."

LC-MS (Thermospray, Fast Atom Bombardment, Particle Beam)

In 1898, the scientists E. Goldstein and W. Wien showed that a beam of positive ions is deflected by electric and magnetic fields. The development of mass spectrometry (MS) goes back to the work of J. J. Thomson in the year 1910. He demonstrated that the noble gas neon consists of two stable isotopes with mass 20 and 22. Fifty years later, this technique was used in organic chemistry for structure determination, determination of the relative molecular mass, and analysis of small amounts of a sample. The fundamental principle of MS is to produce ions from inorganic and organic substances (without destroying them by ionization). Major components of a mass spectrometer are the ion source for the generation of ions, an analyzer for the ion separation/mass selection by electric and/or magnetic fields and the ion detectors of different types such as electron multipliers, Faraday cups, ion-to-photon detectors or scintillation counters (Miller 2009).

Ionization Techniques

The ionization of analytes can be realized thermally, with an electric field or by bombardment with electrons, ions, and photons. The resulting ions from the analytes can be single ions or clusters, ionized molecules, as well as fragments or associates from the ionized molecules. During thermospray ionization (TSI), the analytes within the liquid sample are contained in a capillary, with the end at a temperature of about 200 °C, under pressure in a heated atomizing chamber. Widely used solvents are CH_3CN/H_2O or CH_3OH/H_2O, with an evaporable electrolyte additive

such as 0.1 M CH_3COONH_4. As the liquid leaves the capillary, a nebula of fine drops is formed. Because of the high temperature in the atomizing chamber, the solution media vaporizes. Ions are formed, by the agency of the electrolyte additive, which reach the mass analyzer by a small leak (called a skimmer) in the atomizing chamber. Furthermore, an electric potential is maintained between the skimmer and the repeller (a further component of the atomizing chamber). One main advantage of this technique is that polar and thermolabile substances can attain the gas phase without a direct vaporization process. Problems can arise when the sample is insoluble in the solvent and/or only few solvents are suitable.

Fast atom bombardment (FAB; also called liquid secondary ion mass spectrometry (LSIMS)) belongs to the group of desorption methods. The analytes of the sample are dissolved in a thin, liquid, nonvolatile matrix (e.g., glycerol, 3-nitrobenzyl alcohol, thioglycerol, diethanolamine) that is placed onto a metal plate. The matrix is brought into the ion source and is bombarded with accelerated primary particles in the keV energy range. For this desorption process, inert gases like Xe or Ar are used. During this process, secondary ions are formed which can be accelerated, focused, and analyzed by common methods. Cluster ions from the liquid matrix are also desorbed and produce a chemical background that varies with the matrix used. The FAB technique is gentle and can therefore be used for analysis of proteins and peptides.

Particle beam (also called monodisperse aerosol generator-based interface for liquid chromatography (MAGIC)): the solved analytes are separated with a chromatography column. At the end of the capillary, a helium flow, in combination with TSI or other pneumatic techniques, generates aerosols. The aerosol is sprayed (after focusing the particles into a beam by aerodynamic lenses) into the desolvation chamber where the solvent is vaporized by temperature and low pressure. A fraction of the vaporized particle beam is ionized and diffuses into the mass spectrometer (Smith et al. 2011).

Mass Spectrometer

The ions are separated by their mass-to-charge ratio and recorded with the aid of a detection system according to their mass and count-frequency (qualitative/quantitative). To realize the separation of the ions, static or dynamic electric and magnetic field are used as well as differences in their time of flight. Sector field mass spectrometers and, more commonly, quadrupole mass spectrometer (see Fig. 9) are widely used (Gross 2011).

LC-MS systems are widely used as in the analysis of pesticides, mycotoxins, in the field of clinical chemistry and in forensic analysis (Gergov 2008; Maurer Hans 2010; Botitsi et al. 2011; Roux et al. 2011; Shephard et al. 2011).

LC-MS/MS

LC-MS/MS, also often called tandem MS, is a LC technique with two or more mass spectrometers which are coupled together. This combination increases efficient separation, sensitivity, and selectivity. This kind of mass spectrometry is often used for the analysis of biomolecules (proteins or peptides) and drugs as well as

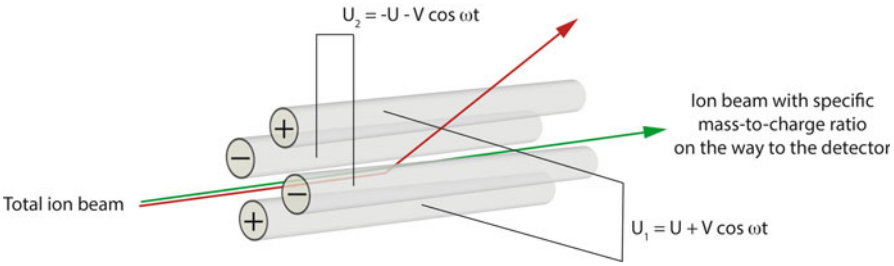

Fig. 9 Scheme of a quadrupole used in quadrupole mass spectrometer. The quadrupole electromagnetic field is adjusted so that only a special mass-to-charge ratio of the total ion beam can pass through the quadrupole (stable ion path; green arrow) and enter the detector system. Ions with another mass-to-charge ratio cannot pass the detector (unstable ion path; red arrow). The potential on the quadrupole, and in consequence the electromagnetic field, is not static. It will change many times per second. Therefore other mass-to-charge ratios reach a stable ion path and can traverse the quadrupole and thus reach the detector system

the study of metabolic pathways (El-Najjar et al. 2017; Adaway et al. 2015; Brandsma et al. 2017; Dong et al. 2018). A widespread variant of the tandem MS is the triple quadrupole MS. This technique is closely related to the chemists R. Yost (1953), C. Enke (1933), and J. Morrison (1924–2013). Their investigations began in the 1970s, and this technique has been widely used since the mid-1980s. Three quadrupoles are used for this MS construction. Usually, the first quadrupole (see section "LC-MS (Thermospray, Fast Atom Bombardment, Particle Beam)" and Fig. 9) is set so that only ions with a certain mass-to-charge ratio can pass through (selection step/selection of the ions). These ions are fragmented in the second quadrupole using a collision gas and transferred to the third quadrupole, where the fragments are separated and, after leaving the quadrupole, detected (see Fig. 10) (Niessen and Falck 2015). By prior separation of the substance mixture (first quadrupole) and subsequent defined fragmentation (second quadrupole), specific fragments (daughter ions) can be measured with the third quadrupole. In other words, specific daughter ions are formed from a mother ion (parent ion). The observation of the masses of the mother and daughter ions is called mass transition. This transition is characteristic for many substances during a certain fragmentation, which increases the analytical sensitivity. The higher sensitivity is of particular interest in drug analytics (e.g., determination of antibiotics from serum, misuse of illegal substances) or the detection of smaller molecules in the ng/l range.

In addition, it is also possible to make smaller molecules out of larger molecules such as proteins by further fragmentation, which can then be detected. It is also possible to add further MS to this cascade.

GC: Including Coupled Techniques

The chemists E. Cremer and F. Prior are among the most important pioneers in gas chromatography (quantitative adsorption-GC with mixtures of gases) (Bobleter 1990). They conceived this technique in 1944. A. Martin and A. James invented

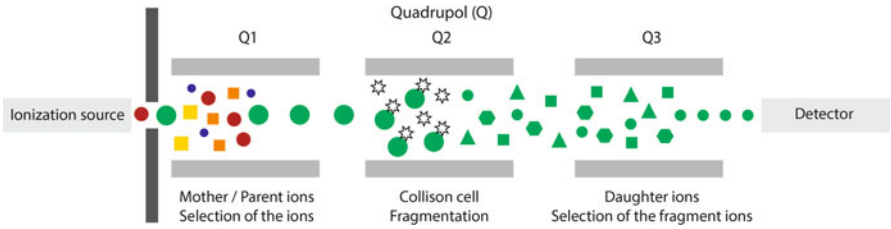

Fig. 10 Schematic diagram of a triple quadrupole mass spectrometer. The ionization of the substances is carried out using techniques such as electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). The first selection takes place in the first quadrupole. Only molecules with a certain mass-to-charge ratio pass through the first quadrupole. These molecules are also called mother/parent ions. They are fragmented in the collision chamber in the second quadrupole. Gases such as argon or nitrogen can be used for fragmentation (symbolized by a star). The fragment ions, which are also called daughter ions, are separated in the third quadrupole according to their mass to charge ratio. Subsequently, the detection takes place, e.g., by means of an electron multiplier

the GC detector in 1952. GC is suitable for chromatography involving the separation of gases or other substances that are vaporizable. The mobile phase is a gas. The stationary phase can be liquid (gas-liquid chromatography, GLC) or solid (gas-solid or adsorption chromatography, GSC). For GLC thin films of a liquid are deposited on a solid particle. Together, this combination builds the stationary phase. It is important to use nonvolatile liquid substances like silicone oil, liquid paraffin, waxes, and polymeric esters. Materials for particles are glass, PTFE powder, diatomaceous earth, or alumina. In adsorption chromatography, the molecules from the sample interact with the solid adsorbent. Typical materials used as adsorbent are aluminum oxide, silica gel, zeolites, or polyamide.

In analytical chemistry, capillary GC, sometimes also called high resolution (HR)-GC, is often used. The capillary columns consist of amorphous sintered quartz (fused silica, FS-columns) stabilized with a thin layer of polyimide. Two different types of capillary columns or Golay columns (named after the inventor) are used: the wall-coated open-tubular column (WCOT-column) and the support-coated open-tubular column (SCOT-column). A WCOT-column can have a length from 5 to 200 m, an inner diameter of 0.1–0.5 mm, and a thin film of separation fluid (stationary phase) at the inner wall of 0.1–0.3 μm. SCOT-columns have an impregnated support material instead of a thin film of fluid as the stationary phase. Special forms of SCOT-columns are the porous-layer open-tubular columns (PLOT-columns). In this case, the stationary phase consists of adsorption material such as aluminum oxide, silica gel, or a molecular sieve. For practical use, the column dimensions (length, diameter, film thickness), as well as the phase composition (such as 10% phenyl polysiloxane) are of interest.

Only compounds with vapor pressures exceeding about 10^{-10} torr can be analyzed by GC. Many compounds with lower pressures can be analyzed if they are chemically derivatized. Derivatization, in this context, is the process of chemically modifying a compound to produce another compound that has properties suitable for

analysis using GC. In most cases the volatility or the stability of the analytes as well as their chromatographic behavior requires improvement. In chemical terms: Derivatization can eliminate polar groups such as OH, NH, COOH, PO_4^{3-}, or SH and therefore increase the volatility and thermal stability of the compound. With steroids and cholesterol, the detectability is increased. GC derivatization methods can be classified into four groups according to the reagents used and the reaction achieved: silylation, acylation, alkylation, and esterification (see section "GC-MS").

Headspace GC

Headspace GC analysis is a special technique for the detection of volatile analytes in the space over fluid or solid samples (see Fig. 11). The sample is put in a gas-tight vial with a septum and heated within the headspace apparatus to a certain temperature. After establishing equilibrium between the sample and the space over the sample, an aliquot from the headspace is analyzed. In routine analytical use, the headspace technique has been applied to the detection of chlorinated hydrocarbon and other (organic) solvents in drinking water, oxbow lake, and wastewater. Moreover the amount of unpolymerized monomers from acrylate, isocyanate, or styrene-based materials can be detected.

This technique has some parallels to the SPME technique (see section "Solid-Phase Microextraction"). But in contrast to that technique, no adsorption material is used. For headspace GC, it is necessary to have an aliquot from a sample to put inside the apparatus. The SPME technique, however, can be used outside the laboratory for onsite measurements.

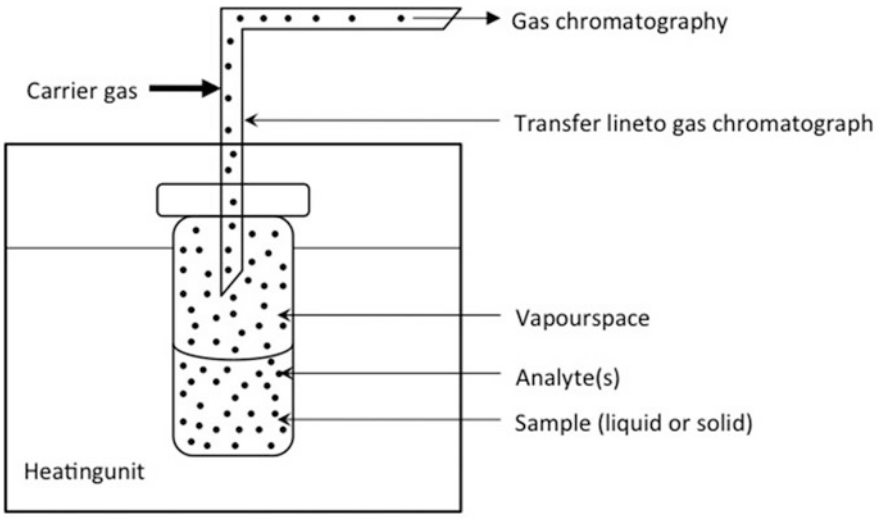

Fig. 11 Scheme of a headspace sampler. A solid or liquid sample is applied to a heating unit (e.g., a water bath). After heating (and waiting), an equilibrium is formed between the analyte(s) in the sample and the analyte(s) in the vapor space (headspace). Aliquots from the headspace are transferred with a carrier gas to a gas chromatography instrument

GC-MS

The great advantage of this combination is that GC can separate volatile and semi-volatile compounds with high resolution, although it cannot identify them. MS can provide detailed structural information on most analytes such that they can be exactly identified, but it cannot readily separate them. After the separation of the analytes by the chromatography column, a mass scan can be obtained. The mass spectrogram of each component is a characteristic fingerprint. To identify a substance, the fingerprints can be compared with mass spectrograms from a database. To quantify the analyte(s), a special mode (selected ion monitoring, SIM) can be used. In this case, the mass spectrometer measures not all masses but only a few analyte typical masses. In this case the sensitivity is increased up to the pg or ng range (Song and Marriott 2012; Watson and Sparkman 2008).

GC-MS systems are used for the identification and quantization of volatile and semi-volatile (organic) compounds and for structural determination (in combination with other techniques) of unknown substances. Common applications are the quantization of pollutants in drinking and wastewater as well as quantization of drugs and their metabolites in blood and urine for pharmacological and forensic reasons. Moreover GC-MS is used for the identification of unknown substances in waste dumps, for the identification of reaction products in synthetic steps, and in quality management for the analysis of industrial products. For a suitable and effective derivatization reaction, some criteria should be considered before choosing the derivatizating reagent:

- High degree of derivatization achievable (90–100%).
- The derivative is stable with respect to time.
- The derivative does not react/destroy the GC column.
- During the derivatization reaction, no rearrangements or structural alterations should occur.
- The derivatization reagent should not induce a loss of analytes during the reaction.

Typical chemical derivatization reagents are:

(a) For silylation, e.g., allyltrimethylsilane, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (both introduce a trimethylsilyl (TMS) group: which is the most popular and versatile silyl group).
(b) For acylation, e.g., trifluoroacetic anhydride, 1-(pentafluoropropionyl) imidazole.
(c) For alkylation, e.g., *N*,*N*-dimethylformamide dimethyl acetal, trimethyloxonium tetrafluoroborate
(d) For esterification, e.g., boron trifluoride, methanol-HCl (Halket and Zaikin 2006; Rosenfeld 2010; Soederholm et al. 2010)

Thin-Layer Chromatography (TLC)

The physical principle of thin-layer chromatography (TLC) is the movement by capillary forces of a liquid phase, usually an organic solvent, through a thin, uniform

layer of solid phase, usually silica gel (SiO_2). The solid phase is held on a rigid or semi-rigid support, normally a glass, aluminum, or plastic sheet or plate. The analytes of the sample are separated by the interaction between the mobile and stationary phases (Spangenberg et al. 2011). Advantages of TLC, especially in the field of forensic analytics, are (Bele and Khale 2011):

- Reliable, rapid, and easy procedure (normal case)
- Relatively inexpensive
- Relatively simple in use
- Possibility of detecting upward of 700 different types of drugs, medications, and metabolites
- Validated as a diagnostic tool that holds up under the scrutiny of legal challenges, inside and outside the courtroom
- Combined with sample pretreatment (e.g., solvent extraction) TLC can be a powerful qualitative technique

It should be mentioned that the interpretation of TLC results is sometimes very difficult, especially when a number of drugs, medications, and metabolites are present.

TLC can detect, accurately, a large number of medically significant substances, such as anticonvulsants/antispasmodics (e.g., phenytoin, carbamazepine), antidepressants (e.g., amitriptyline, nortriptyline, sertraline), antihistamines (e.g., chlorpheniramine, diphenhydramine), anti-inflammatories (e.g., naproxen, ketoprofen, ibuprofen), anesthetics (e.g., lidocaine, procaine), decongestants/broncodilators (e.g., ephedrine, theophylline), muscle relaxants (e.g., carisoprodol, meprobamate), narcotic analgesics (e.g., methadone, tramadol), sedatives (e.g., ketamine, imipramine), stimulants (e.g., methylphenidate, methylenedioxy-methamphetamine (MDMA)), and miscellaneous (e.g., strychnine, verapamil, haloperidol) (Parmar et al. 2011; Shewiyo et al. 2012; Tuzimski 2011).

Ion Exchange Chromatography (IEC/IEX)

IEC is a distinctive kind of adsorption chromatography, which allows separation of ions. It has a special significance in the analysis of organic and inorganic ions such as phosphate (PO_4^{3-}) or sulfate (SO_4^{2-}). Ion separation is based on the charge and the size of each analyte ion itself as well as the counter-ions, the pH, and the ionic strength in the mobile phase and the type of exchange resin. The stationary phase is an ion-exchange resin. Ion-exchange resins are categorized as cation and anion exchangers. In both classes, strong and weak ion exchange resins exist (see Table 3). Liquid-phase analyte ions are attracted via Coulombic forces to ions in the exchange resins (stationary phase). Elution of the analyte ions occurs by an exchange with an ion from the eluent (Inamuddin and Luqman 2012a, b).

IEC is used in many fields where small molecules/ions must be detected; for some molecules/ions, it is the preferred method. Here are some examples: In the field of environmental analytics, it can be used for the detection of inorganic anions such as nitrate, nitrite, bromide, fluoride, chloride, or inorganic cations like Cr(VI), Ni^{2+},

Table 3 Classification of ion exchange resins and their chemical functional groups

Ion exchange resin	Functional group	
Cationic exchanger		
Strong acid	Sulfonic acid	$-SO_3H$
	Phosphoric acid	$-PO(OH)_2$
Weak acid	Hydroxyl group	-OH
	Carboxyl group	-COOH
Anionic exchanger		
Strong alkaline	Quarternary amine	$-N^+(CH_3)_3$
	Diethylaminoethyl-(DEAE)	$-(CH_2)_2-N^+H(C_2H_5)_2$
Weak alkaline	Primary amine	$-NH_2$
	Secondary amine	-NH-
	Tertiary amine	$-\underset{\vert}{N}-$
	Chelating resins (aminophosphonic acid, iminodiactic acid, thiols	$NH_2CH_2PO(OH)_2$ NH $(CH_2COOH)_2$ -SH

Cu^{2+}, or metals in complexes such as $Au(CN)^{2-}$ and $Au(CN)^{4-}$ from complex matrices. In the field of food analytics, organic molecules such as pyruvate, lactate, citrate, or amino acids can be measured (Inamuddin and Luqman 2012a, b; Karlsson and Hirsh 2011).

Electrophoresis

The electrokinetic phenomenon was observed in 1807 by the German scientist F. Reuss at Moscow University (Reuss 1809). Electrophoresis, as known today, was first described in 1937 by the Swedish chemist A. Tiselius. The term derives from the Greek words "ηλεκτρου, electron" "electron" and "φορεσις, phoresis" "carrying" meaning "electric carried." The physical principles of electrophoresis are based on the motion of analytes (cells/particles/proteins/substances) relative to a fluid under the influence of a spatially uniform electric field (see Fig. 12). The migration speed and in consequence the retention time of the analytes depend on their charge, mass, and size as well as the electrophoresis media and the strength of the electric field. The results are singular bands visualized on a gel, a foil, or an electropherogram (e.g., presentation of DNA sequencing). Modifications of electrophoresis are, for example, slab-gel electrophoresis and capillary electrophoresis (CE). CE (also known as capillary zone electrophoresis, CZE) uses – besides the normal electrophoresis conditions (conductive liquid medium under the influence of an electric field) –

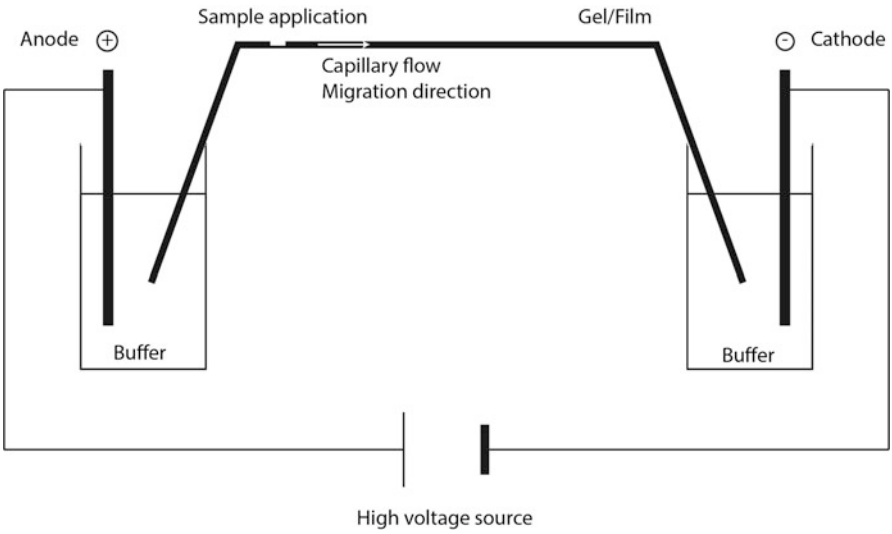

Fig. 12 Principle of electrophoresis. The sample with the macromolecules is applied on a film/gel, e.g., a cellulose acetate gel. Then an electric field is applied. The charged molecules migrate toward the positive or negative pole according to their (opposite) charge (in the Figure, the positive macromolecules migrate to the cathode). By passing through the film, the macromolecules are separated by their size, charge, and conformation. After a certain time, the electrophoresis is stopped and the macromolecules are stained (discontinuous working system). The gel is only for single use. In contrast to electrophoresis, capillary electrophoresis is a continuous working system. The separated macromolecules are detected by a UV-detector, or a similar device

electroosmosis, as a further separation principle. If ions are in the solution medium, then an electroosmotic flow will be generated with the negatively charged analytes and the negatively charged ions moving to the anode. The positive ions in the medium as well as the analytes (independent of the charge) flow to the cathode. A short overview of the application fields and possibilities are given in (Pascali et al. 2012; Harrington et al. 2010; Simpson et al. 2008).

Atomic Spectroscopy

Atomic spectroscopy embraces a set of spectro-analytical techniques for the qualitative and quantitative determination of chemical elements (Hywel Evans et al. 2012). J. Marci von Kronland described in 1648 the diffraction and the scattering of light in waterdrops. The first spectroscope, consisting of a lens, a prism, and a screen, to define a light beam, was developed by I. Newton in 1666. He showed that the white light from the sun could be dispersed into a continuous series of colors (a light spectrum). In 1752, T. Melville discovered that putting different substances/salts in flames, and passing the light through a prism, leads to different spectra. He

found that table salt generated a "bright yellow." In 1815, J. Fraunhofer discovered in the optical spectrum of the Sun dark features (absorption lines). In 1853, A. Angstrom analyzed the spectrum of hydrogen and obtained the first insight into atomic structure. In the years from 1855 to 1863, R. Bunsen and G. Kirchhoff systematically investigated thousands of spectral lines (Burns 1975; Thomsen 2006). The first element discovered by spectral lines was helium. The first hint of the existence of helium was seen in 1868 by the astronomer J. Janssen. He saw a bright yellow line with a wavelength of 587.49 nm in the spectrum of the chromospheres of the Sun (Tayler 1995). The principles of quantum theory, interrelating atomic structure with electromagnetic radiation, were initiated by M. Planck, about 1900.

The principle of all atomic spectroscopy techniques is based on the characteristic behavior of atoms/elements (but not chemical compounds) that under certain physical circumstances an *element-specific* electromagnetic emission or absorption takes place (Bings et al. 2010). This interaction corresponds to a change of the energy state of the outer electrons of each atom. For this reason, the analyte elements in the sample have to be released from their compounds. Free atoms can be generated by atomization in a flame or plasma. Widely used atomic spectroscopy techniques are:

- Atomic absorption spectroscopy (AAS)
- Atomic emission spectroscopy (AES)
- Atomic fluorescence spectroscopy (AFS)
- Optical emission spectroscopy (OES)
- Inductively coupled plasma mass spectrometry (ICP-MS)
- X-ray fluorescence spectroscopy (XRF spectroscopy)

The interaction between the outer electrons of the atom and electromagnetic radiation can involve atomic absorption, atomic emission, and atomic fluorescence. Atomic absorption occurs when they absorb ultraviolet (UV) and/or visible light (VIS) radiation. The unabsorbed radiation is measured. The atoms reach an excited state (higher orbital) from the ground state. In the case of atomic emission, the excited electrons (e.g., after thermal or electronic excitation) revert to the ground state by emission of electromagnetic radiation. With fluorescence spectra, the atoms are excited with light or laser, and then light of a longer wavelength range is emitted and measured.

In *qualitative* atomic spectroscopy, the characteristic lines (wavelengths) for each element are measured. In *quantitative* atomic spectroscopy, the intensity of the lines from each element is determined, and the amount of this element is calculated with the aid of a calibration line. The quantification relies on the Beer-Lambert law. With different atomic spectroscopy techniques, the following (most relevant) elements that can be measured are Ag, Al, As, Au, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs,Cu, Dy, Er, Eu, Fe, Ga, Gd, Ge, Hf, Hg, Ho, In, Ir, K, La, Li, Lu, Mg, Mn, Mo, Na, Nb, Nd, Ni, Os, P, Pb, Pd, Pr, Pt, Rb, Re, Rh, Ru, S, Sb, Sc, Se, Si, Sm, Sn, Sr, Ta, Tb, Te, Th, Ti, Tl, Tm, U, V, W, X, Y, Yb, Zn, and Zr (Welz et al. 2005; Skoog et al. 2018).

Atomic Absorption Spectroscopy (AAS)

This technique is based on the absorption of optical radiation by free atoms in the gaseous state. AAS can be used to determine over 70 different elements in solution or in solid samples. AAS was developed in the 1950s by the team led by A. Walsh. The first stage of AAS requires atomization of the sample analytes in the atomizer. Then the atoms are irradiated by optical radiation. To obtain an exact element-specific wavelength for each element, the radiation has to pass a monochromator. The element-specific radiation is measured and the signal amplified within the detection system (see Fig. 13) (Welz and Sperling 2007).

Within the general technique of atomization, different atomizers are available: Flame atomic absorption spectroscopy (FAAS), electrothermal atomic absorption spectroscopy (ETAAS), graphite furnace atomic absorption spectroscopy (GFAAS), and cold-vapor atomic absorption spectroscopy (CVAAS). In the case of FAAS, a combination of a burning gas and an oxidation gas is used. The combination depends on the analyte elements (poorly vaporable elements like Mg, Ca, W needs higher temperatures). Usual combinations include acetylene (burning gas) and nitrous oxide (N_2O; oxidation gas), which reaches temperatures of about 3,200 K, or acetylene and oxygen, reaching temperatures of about 3,000 K. In the case of GFAAS, the liquid, solid, or gaseous sample can be analyzed directly. The sample is put in the graphite tube, and a temperature program is started leading to drying, pyrolysis, and atomization of the sample. As a final step, the graphite tube is cleaned at high temperature.

Different lamps are used as radiation sources. First, it is necessary to distinguish between two types of lamps: line source (LS) and continuum source (CS) lamps. LS lamps emit a single line spectrum. CS lamps emit continuous spectra. In classical AAS, CS lamps like deuterium hollow cathode lamps (HCL) where used for

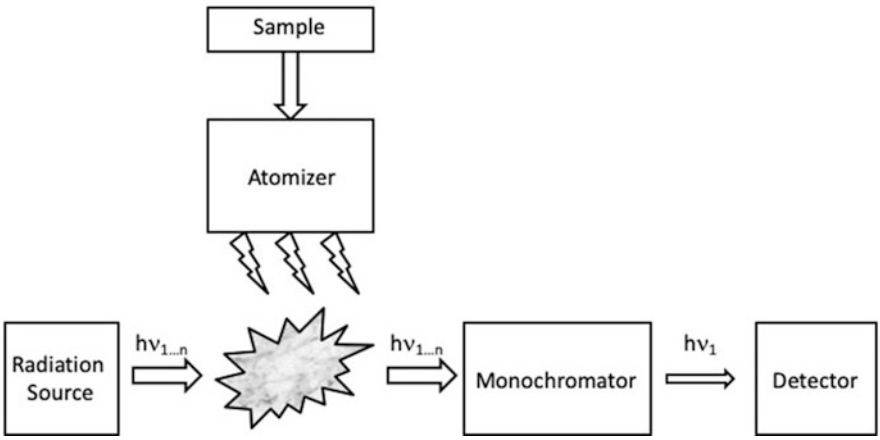

Fig. 13 Schematic description of atomic absorption spectroscopy (AAS). The elements in the sample are atomized. The elements are activated by a radiation source. The emitted spectra from the elements are, after passing a monochromator, detected, multiplied, and evaluated

background compensation. Newer developments, such as high-resolution continuum source atomic absorption spectroscopy (HR-CS AAS), use CS lamps like xenon compact-source arc discharge lamps. These provide a high radiation density and cover the complete spectral range from the near vacuum-UV to the near infrared.

For LS AAS, normally HCL are used. HCL consist of a glass tube containing a cathode, an anode, and a buffer gas (usually a noble gas). The cathode is made from the element to be analyzed. The high voltage between the anode and cathode ionizes the buffer gas (a plasma is created). The gas ions are accelerated towards the cathode, sputtering off atoms from the cathode. The sputtered atoms from the cathode will be excited by collision with other particles in the plasma. By decaying to lower energy states, these excited atoms emit photons, which are used for identifying the element in the sample (Karabegov 2011; Kumar et al. 2009; Ataman 2008).

Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

In the early 1980s, the commercialization of ICP-MS started. Today, many different ICP-MS instruments are commercially available, each with their own strengths and limitations. They all share similar components such as the nebulizer, spray chamber, plasma torch, interface, and detector but can differ significantly in the design of the mass spectrometer and in particular the mass-separation device (Nelms 2005).

ICP-MS is based on the ionization of sample elements in a plasma at about 5,000 to 10,000 K. Normally the plasma is produced by the interaction of an intense magnetic field (produced by radiofrequency radiation passing through a copper coil) on a tangential flow of gas (normally argon), flowing through a concentric quartz tube (torch) at about 15 L/min. This setup ionizes the gas and, when seeded with a source of electrons from a high-voltage spark, forms a very high temperature plasma discharge (~10,000 K) at the open end of the tube.

The sample, typically in liquid form, is pumped into the sample introduction system, which is made up of a spray chamber and nebulizer. It emerges as an aerosol and eventually passes – by way of a sample injector – into the base of the plasma. As it travels through the different heating zones of the plasma torch, it is dried, vaporized, atomized, and ionized. During this time, the sample is transformed from a liquid aerosol to solid particles, then into a gas. When it finally arrives at the analytical zone of the plasma, at approximately 6,000 to 7,000 K, it exists as excited atoms and ions, representing the elemental composition of the sample.

In the next step, the ions are directed into the mass spectrometer via the interface region. The role of the interface is to transport the ions (and only the ions) from the plasma, which is at atmospheric pressure (760 torr) to the mass spectrometer analyzer region at approximately 10^{-5} torr. Moreover the interface has to reduce or eliminate the secondary discharge, which arises by capacitive coupling between the radiofrequency coil and the plasma. After the interface the ion optic (a series of electrostatic lenses) focuses the ion beam toward the mass separation device, and it

stops photons (that would otherwise increase the signal noise), particulates, and neutral species from reaching the detection system. The most common types of mass separation devices are based on quadrupole, magnetic sector, time of flight, collision/reaction cells, and dynamic reaction cell technology. The basic principle of these different types of mass separation devices is to allow only analyte ions of a particular mass-to-charge ratio (m/z) to pass the device and to fly to the detection system. Other particles such as matrix ions have to be filtered out. At last, the ion detector converts the ion beam into an electrical signal. Widely used are dynode detector systems, containing a series of metal dynodes along the length of the detector. In this case the ion beam impinges upon the first dynode and creates an electron beam, which attracts the next dynode. The process of electron multiplication starts.

One great advantage of this technique is its ability to carry out rapid multi-element determinations at low detection limits (ultra-trace level), especially enhancing the speed of analysis and the isotopic capabilities (Aggarwal 2010; Butler et al. 2010).

Selective Analytical Chemistry

Sensor Techniques

The term sensor techniques subsume different molecule measuring techniques. Biosensors are widely used with their special form of ion-selective electrodes (ISE), for detecting supramolecular interactions on interfaces. According to the International Union of Pure and Applied Chemistry (IUPAC), a biosensor is defined as "a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in direct spatial contact with a transduction element" (Turdean 2011). Biosensor techniques are based on direct measurement of a (biological) component with the aid of a biorecognition phases, such as enzymes, antibodies (immunosensors), single-stranded DNA, or microorganisms (whole-cell based biosensors). The analyte interacts with the biorecognition phase and produces a signal, e.g., a change in proton concentration; a release or uptake of gases like oxygen; a release or uptake of electrons; a light emission, absorption, or reflectance; a heat emission; or a mass change. For example, an antigen can be detected by coupling with an antibody. The antibody is directly coupled with a transducer, transforming the chemical signal into a measurement signal/measurable response. This type of system is quite general. Transducers can be electrodes based on an optical fiber, a piezoelectric crystal, electrochemical methods (potentiometric or amperometric systems), sonic methods, or a calorimetrical system (thermistor). The signal from the transducer is electronically processed, and the measurement value is displayed (see Fig. 14) (Farré and Barceló 2009).

One classification of biosensors is based on the detection system of the transducer. Biosensors are used in ecotoxicology (formaldehyde detection) and

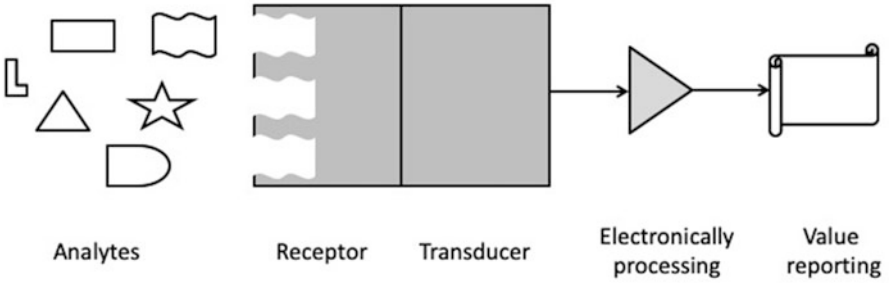

Fig. 14 Principal construction of a biosensor. An analyte-specific detection system is used, which is coupled with a transducer. The transducer converts the chemical signal from the detection system into an electronic signal that is amplified, and the value obtained is reported

environmental monitoring (pesticides, nitrites) as well as for breath tests, in food control measurements ("artificial nose" to determine freshness, aroma, odor) and for military use, e.g., detection of nerve gas and chemical or biological weapons.

ISE is a form of potentiometry. That means a special from of electrochemical-based biosensors that determine the equilibrium cell voltage of galvanic cells. ISE measure the activity of a special analyte ion in a solution of different ions. This produces a potential that is proportional to the concentration of the analyte ion. ISE are used for measuring in brass, bronze, copper, lead, and cadmium baths as well as for the determination of ethylenediaminetetracetate (EDTA) and citrate. Dependent on the ion-selective membrane, solid-state and liquid membranes are distinguished. Solid-state membranes can be based on glass membranes, single crystal membranes, or precipitation membranes. Liquid membranes can be based on ion carrier membranes or ion exchange membranes (Gruendler 2007).

Immunoassays

Immunoassays (IAs) are widely used laboratory methods for clinical and (forensic) toxicology diagnostics. IAs are useful for blood or serum therapeutic drug monitoring. They are also useful for serum and urine determinations of ethanol, medicines, drugs of abuse (amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine, ecstasy, methadone, opiates, phencyclidine, tricyclic antidepressants), and other toxins. The basic principle of the IAs is the detection of the analyte (in this case "the antigen") in a liquid phase by bonding with an antibody (antigen-antibody reaction, see Fig. 15). Normally, IAs are based on a competitive and cooperative interaction between the analyte (a hormone, a protein, a drug or a hapten) to be determined and a labeled ligand, which is thus measurable, and an unlabeled ligand, both of which occupy the same binding site on the analyte. The labeling can be achieved with a fluorescent dye, a luminogen, a fluorophor (FIA), an enzyme (EIA), or a radioactive (RIA) substance. IAs are fast, sensitive, and accurate and permit

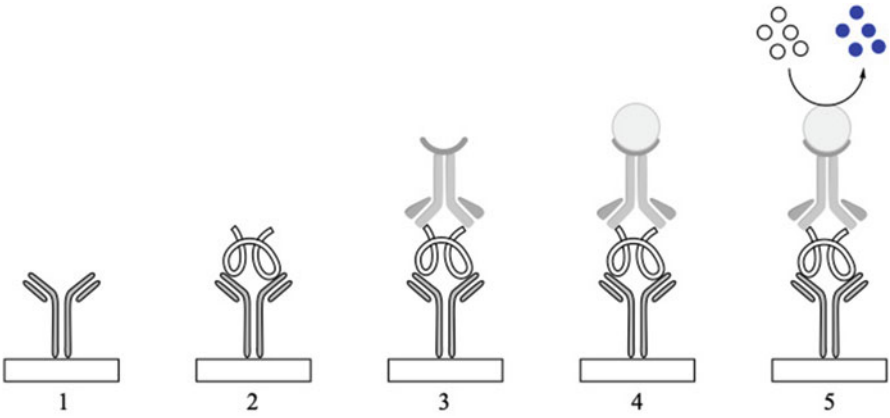

Fig. 15 Principles of an enzyme-linked immunosorbent assay (ELISA). (1) Analyte-specific antigens are bound to the wall of a reaction vessel. (2) After adding the sample, the analyte binds to the analyte-specific antibody. (3) After washing, a second biotin-labeled antibody binds to the analyte. (4) After washing, a streptavidin-enzyme conjugate binds to the biotin-labeled antibody. (5) The streptavidin-enzyme conjugate catalyzes the formation of a chromogen from a colorless substance that is added

determination of analytes in different kinds of (biological) fluids or suspensions (Durner 2010; Moody 2006; Chan et al. 2008). One problem is cross-reactivity with additional matrix components, such as metabolites or structurally related substances.

IAs can be classified by different criteria. Widely used is the classification depending on their realization. In this case, homogeneous IA scan be distinguished from heterogeneous. In contrast to homogeneous IAs, the unbound reactants are separated prior to measurement in heterogeneous IAs. In the case of heterogeneous IAs, two further types can be distinguished, a competitive IA and an immunometric IA, also known as "two-site" or sandwich IA. A very common form of sandwich IA is the enzyme-linked immunosorbent assay (ELISA). In ELISA test systems, the antigen in the liquid sample is captured by immobilized antibodies (e.g., on the wall of cavities in 96-well plates or on polystyrene globes). After washing steps, a second, enzyme-labeled antibody (e.g., with horse radish peroxidase, alkaline phosphastase, b-galactosidase) against the antigen is added. Then a substrate is added which is converted to a chromogenic reaction product if the enzyme from the antibody (and therefore the analyte) is present in the reaction vessel. The concentration of the analyte can then be determined through absorption spectroscopy according to the Lambert-Beer law.

In general, homogeneous IAs are more amenable to full automation and thereby quicker throughput. Heterogeneous IAs are less susceptible to matrix interference and thereby more versatile with non-urine matrices.

Other common IAs are cassette or strip rapid tests like lateral flow immunoassays (LFA) (Christopher et al. 2005; Posthuma-Trumpie et al. 2009). Such tests are used

in environmental analytics (water testing, pesticides, dust mite testing), food testing (genetically modified (gm) food, *Escherichia coli*, *Salmonella* strains), military analytic (germ warfare, explosives chemical warfare), veterinary analytics (feline cancer, BSE, canine heart worm), disease diagnostics (malaria, hepatitis B, tuberculosis), testing of sexual transmitted diseases (STDs; *Chlamydia*, syphilis, HIV), fertility diagnostic (pregnancy, luteinizing hormone), or drug abuse (cocaine, cannabis, ecstasy).

Special Types of Mass Spectrometers

The very widely used quadrupole mass spectrometers were discussed in the chapter on separation techniques, as they are often combined with a GC or HPLC/LC and are used for routine investigations (see section "LC-MS (Thermospray, Fast Atom Bombardment, Particle Beam)"). At this point, a special type of mass spectroscopy will be discussed, which is mainly used in research.

(LC)-MALDI-TOF

MALDI-TOF-MS is one of the newer methods in the field of analytical chemistry. The technique was developed in the 1980s by M. Karas and F. Hillenkamp and also K. Tanaka et al. (Karas and Hillenkamp 1988; Tanaka et al. 1988). It is a discontinuous method that produces ions after exposure to a laser beam. Ion generation, acceleration, and mass analysis can be repeated in short time intervals. It is a suitable method for protein, peptide, oligonucleotide, synthetic polymer, and organic macromolecule measurements as well as for bacterial identification in clinical microbiology (Seng et al. 2010; Kafka et al. 2011; Li et al. 2010; Oeth et al. 2009; Vestal 2009). The sample is mixed with a matrix. This consists of low-molecular mass organic substances such as all-*trans*-retinoic acid, 2,5-dihydroxybenzoic acid, 5-hydroxysalicylic acid, or 9-nitroanthracene which have an absorption maximum at a laser wavelength of 337 nm. This mixture is presented on a sample plate. Then the solvent is evaporated, the plate inserted within the sample chamber of the MS and bombarded with the laser beam (discontinuous in the range of ns). Then an acceleration voltage is applied to accelerate the ions to the detector. The kinetic energy from each ion is equal. Their velocity depends on their mass-to-charge ratio. After detection, the mass of the ions can be determined by their time of flight. In some MALDI-TOF, mass spectrometers reflectors are inserted. A reflector generates a multilevel electric field. The ions are reflected from their trajectory and registered by a second detector. Using this technique, it is possible to compensate for smaller kinetic energy differences from ions equal in mass (see Fig. 16). Faster ions plunge deeper into the electric field of the reflector and stay there longer (Mamyrin 1994). By using this "trampoline effect," it is possible to reduce the mass resolution limit many times over.

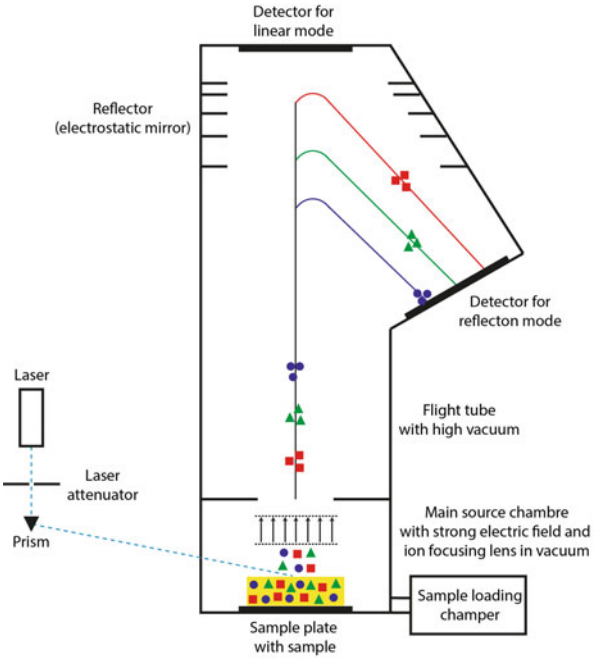

Fig. 16 MALDI-TOF MS schematic. A laser beam ionizes the analyte(s) from the sample plate which are accelerated in the electric field. In the linear mode, the analyte ions are separated by their mass-to-charge ratio (m/z) in the time of flight tube. The higher the molecular mass the lower the velocity in the tube. During the flight, the analyte ions can be decomposed (post source decay; PSD). In the linear mode it is not possible to separate these PSD ions. By using the reflection mode, uncharged molecules pass the reflector, whereas charged ions will be deflected to a "V"-like path by different potentials in the reflector. Thereby a further separation of ions takes place

Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR-MS)

Cyclotrons are circular particle accelerators in which ions are accelerated within a magnetic field into a spiral-shaped path to trigger, for example, nuclear reactions. A prominent application is the production of isotopes for medical diagnostics such as positron emission tomography (PET). The principle of this ion acceleration is also used in FT-ICT-MS. To arrive at this high-resolution spectroscopy method, basic work on particle accelerators had to be completed first. In the 1920s, there were numerous considerations about building a cyclotron, but these were not implemented. Inspired by a publication on particle acceleration, E. Lawrence (1901–1958) realized that a linear accelerator would require too large dimensions, especially length, for the generation of ions with high kinetic energy. He proposed to develop a more compact design by using circular acceleration. In 1930, he built a cyclotron and produced ions with high kinetic energy (Lawrence and Edlefson 1930; Lawrence and Livingston 1932). The next milestone was the introduction of the

Omegatron by Sommer in 1950 (Sommer et al. 1950). The Omegatron was based on the principles of the cyclotron. However, it was much more compact (approximately the size of a cigarette packet). The advantage of this invention was that the electron/proton mass ratio could be determined physically (and not electrochemically, as before) and with high accuracy for the first time. Also it became possible for the first time to determine the masses of gases and vapors with high resolution (National Bureau of Standards 1950). The now common Fourier transform technique was introduced in 1974 by Marshall and Comisarow and is based on a pulsed measuring method (Comisarow and Marshall 1974).

In this overview, only the basic function and design of FT-ICR-MS can be described. There may be major differences between the various manufacturers. Central components are a strong magnet (up to 21 Tesla) and an ICR trap. This is a modified, advanced Penning trap: that is an ion trap that can hold ions by means of a strong magnetic field. Depending on the geometric shape, there are cubic or cylindrical ICR traps (Fig. 17).

The traps consist of six pairs of electrodes/plates, two axial and four radial. The axial plates are used to trap the ions, similar to creating an electrostatic field in a quadrupole mass spectrometer. The two pairs of radial plates serve on the one hand to excite the ions (excitation plates) and on the other hand to detect the current induced by the ions (detection plates).

By modification by the electrostatic field created by the trapping plates in combination with the static magnetic field, the ions are trapped. To a first approximation, in accordance with the Lorentz force, the ions move in the ICR trap within the magnetic field on a circular path with the so-called cyclotron frequency. The radius of this resting movement of the ions depends on their thermal energy as well as on the strength of the magnetic field (the stronger the magnetic field, the smaller the radius). By emitting radiofrequencies (RF) through the excitation plates (RF generator), the ions can be excited when their cyclotron frequency is in resonance

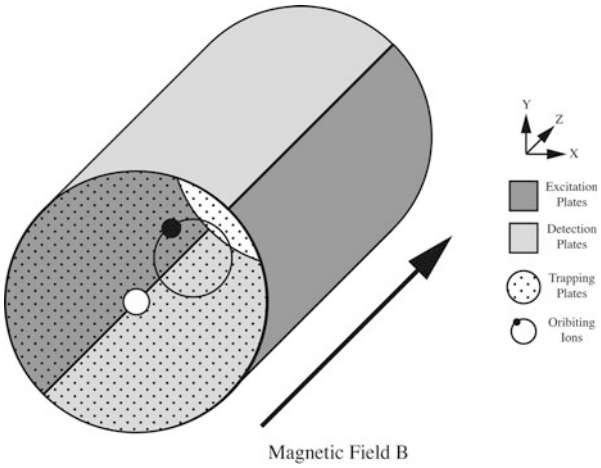

Fig. 17 Cylindrical ICR trap. The two trapping plates are attached axially (dotted area with an inlet for the ions in the middle). Both the excitation plates (light grey) and the detection plates (dark grey) are mounted radially. The ICR cell is located in a strong magnetic field B that points in the axial direction

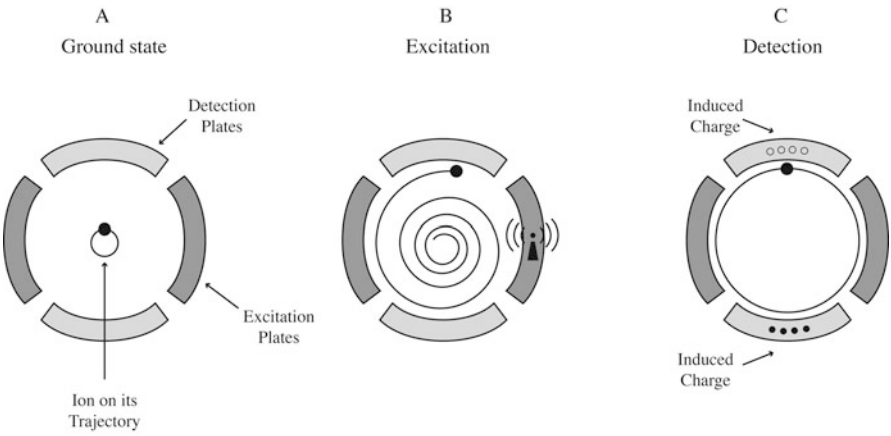

Fig. 18 FT-ICR-MS principle. (**a**) Ions move within a magnetic field on a circular path because they are affected by the Lorentz force. The direction of movement is perpendicular to the magnetic field. The orbital frequency is called the cyclotron frequency. (**b**) Ions can be excited in resonance by irradiation with an alternating radio frequency. (**c**) The excited ions move close to the detection plates and thus induce a measureable voltage

with the corresponding wavelength of the RF (Gross 2017). Their orbital radius becomes increasingly greater. If the radius becomes so large that they are close to the detection plates, it induces a detectable voltage (see Fig. 18). The strength of the induced voltage depends on the number of ions and the distance to the detector plates (orbit radius) and is independent of the mass of the ions. The detected signal is converted into a frequency spectrum via a Fourier transform. From this the mass spectrum is calculated (see Fig. 19), for which a prior external calibration with known masses is required (Marshall and Chen 2015; Pico 2015). One advantage of FT-ICR-MS is that it delivers the greatest resolution and mass accuracy. With classical mass spectrometry, masses in the range between 100 and 1,000 u (u = **u**nified atomic mass unit; it is defined as 1/12 of the mass of a neutral atom of carbon isotope ^{12}C: unbound, in the electronic ground state, at rest) can be resolved very well. With the FT-ICR-MS, measurements over 1,000,000 u can be resolved. One reason for this is that frequencies can currently be determined with higher accuracy than any other physical parameter. However, the FT-ICR-MS devices are large, heavy, and expensive.

The FT-ICR-MS has a wide range of applications. The main focus is for petroleum analytics and the "omic" sciences (such as proteomics, metabolomics) (Zhao et al. 2016; Cho et al. 2015; Ghaste et al. 2016). This technology is increasingly finding its way into pharmacology and toxicology. Pharmaceutical companies often buy the active ingredients for their preparations on the international market. In addition to the active ingredient, the product may also contain adulterants (unethical manufacturers are unfortunately also among the producers of the active ingredients). Due to the increasing demand for phosphodiesterase type 5 (PDE-5), enzyme inhibitors like sildenafil for the treatment of erectile dysfunction in men, counterfeits are available.

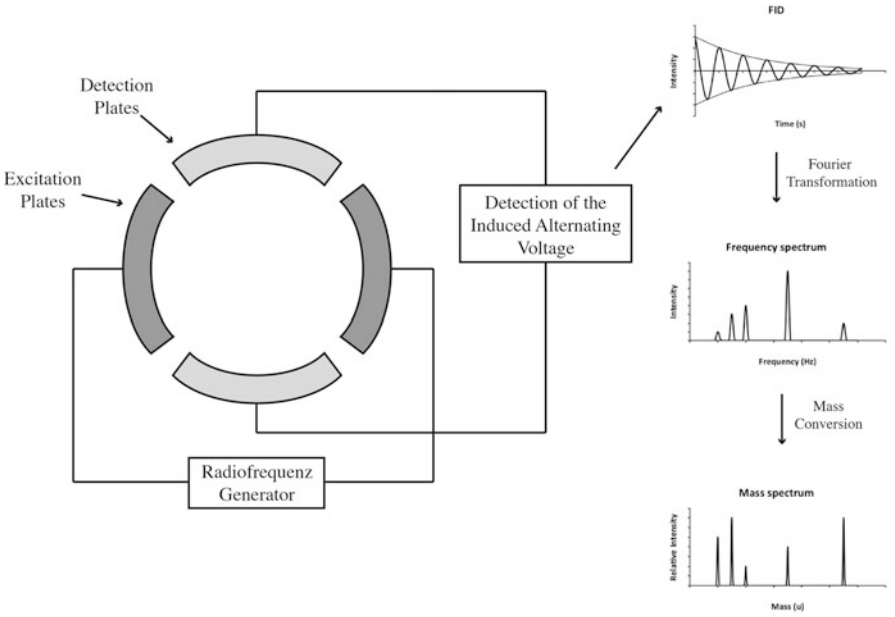

Fig. 19 FT-ICR-MS detection. The voltage induced at the detection plates is measured and converted into a frequency spectrum via Fourier transformation. The mass spectrum is obtained from this after conversion and calibration with standards

More than 50 unauthorized analogues of PDE-5 inhibitors have been described in the literature (Patel et al. 2014). The counterfeiters of the active ingredients constantly synthesize new, cheaper analogues, in the expectation that they will be unnoticed by the normal analytical controls of the buyers. By using new and different analytical techniques, these contaminated and stretched active ingredients can be identified (Patel et al. 2014). In geochemistry, this technique is used to investigate dissolved organic matter (DOM). DOM are dissolved organic substances in the soil with a particle diameter <0.45 μm, which are important for the nutrient cycle and biogeochemical processes in both aquatic and terrestrial environments. It is said that DOM is one of the most mobile and actively cycling organic matter fractions (Bolan et al. 2011). Anthropogenic pollutants can accumulate and be made bioavailable. It has been shown that the release of nano-titanium dioxide (Nano-TiO_2) into the aquatic environment increases the consumption of DOM and reduces its molecular diversity (Lv et al. 2017). Other research groups study the composition of DOM under different conditions and locations. It has been shown that the pH of forest soil has a greater influence on DOM than its temperature (Roth et al. 2015). Other fields of application include the investigation of posttranslational modifications such as methylation of histones to control chromatin function (Starkova et al. 2017) and the influence of chronic alcohol abuse on posttranslational acetylation of histones and cytokine release. In comparison to controls, alcohol abuse causes a significant increase in acetylation of histone H4 at lysine 12

(H4K12ac), which in turn intervenes in the inflammation cascade (Parira et al. 2017). In neuroscience, the hormones of habenular nuclei (a pair of small symmetrical structures in the epithalamus) have been investigated. At the medial core, 27 prohormones were identified; and at the lateral core 20, which in turn were split into 262 and 177 neuropeptides (Yang et al. 2018). FT-ICR-MS can also be used to search for pharmacological active substances in various plant sources. In one algae species, substances have been found that inhibit the reverse transcriptase of HIV-1. Using FT-ICR-MS and database analysis, a structural analysis of these substances has been carried out (Kremb et al. 2017).

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Uncertainty Analysis in Exposure Assessment-Relevance for Toxicological Risk Assessment

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Michael Schümann, Haluk Özkaynak, and Alexandre Zenié

Contents

Abstract

Within the last decade the documentation of uncertainty has become mandatory as a necessary part of any exposure and risk assessment. A key document that is used as a framework in many regulatory approaches is the guidance document published by the WHO (IPCS) in 2008. The structure of this chapter follows the guiding principles described there, adding information from various regulatory documents. The process of an exposure assessment is structured by the definition

M. Schümann (✉)
Department of Health and Consumer Protection, Hamburg, Germany
e-mail: Michael.Schuemann@hamburg.de

H. Özkaynak
U.S. Environmental Protection Agency (U.S. EPA), Durham, NC, USA

A. Zenié
Joint Research Centre, European Commission, Ispra, Italy
e-mail: alexandre.zenie@ec.europa.eu

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of the scope of analysis, the selection of appropriate exposure scenarios for the population under concern, and the choice of conceptual and mathematical models with appropriate parameters. The evaluation of the resulting exposure calculation should support conclusions about the likelihood of exceeding health-based guidance values. The choice of parameters must cover the existing variation of all influence factors. The process should start with simplified approaches and repeated iteratively until the level of residual uncertainty can be tolerated with respect to the purpose. Each uncertainty may be analyzed at one of three tiers: qualitative, deterministic, or variance based. The identification and evaluation of the different kinds and sources of inherent uncertainty is part of the overall analysis and documentation. By this, uncertainty analysis strongly supports informed decision-making and risk communication under uncertainty.

Keywords

Biomonitoring · Limit value · Mathematical model · Regulatory toxicology · Risk assessment · Risk management · Pollution · Scenario · Variability

Introduction

Communicating the results of an exposure assessment that is based on model assumptions and numerical estimates is demanding, communicating the inherent uncertainties at the same time makes the task complex. Any exposure analysis relies on information on the concentrations of a pollutant in an exposure media, on the circumstances and the human behavior and the activities that result in contact and exposure, as well as on the transfer rates from the exposure media to the individual. Exposure increases the internal dose when the agent is transferred into and taken up by the body. Any exposure assessment includes knowledge and assumptions with respect to appropriate exposure scenarios, in relation to the models that should reflect the selected exposure scenarios and with regard to the type and quality of available data that characterizes the exposure conditions described for a population or a subgroup of concern. Risks cannot be reliably estimated if exposures are not properly characterized and, if necessary, sufficiently quantified (IPCS-WHO 2008). Any risk quantification relies on good measurement or appropriate estimates of influential variables. Since valid exposure assessment is a core element in quantitative risk assessment, any inherent uncertainty will influence the quality of results.

Risk assessment results are predictions of the frequency and severity of effects on the ground of exposure estimates. The quality of any risk assessment, and in consequence risk management and risk communication (NRC 1994), are directly dependent on the quality of the exposure assessment process. Risk reduction is often achieved through regulation, enhancement of existing rules and laws that should result in exposure mitigation. For all regulatory purposes, sufficient knowledge about exposure conditions is a basic prerequisite for characterizing subsequent risk

management strategies. For all situations which might contribute to an exposure, the possible active sources, the relevant pathways, and the behavior patterns that contribute to exposure must be identified. The role of exposure assessment is to provide information about the distribution of expected total magnitude of exposure, about the nature of the source, about the routes of exposure, and about the individuals who are exposed. Uncertainty in risk assessment is defined by IPCS (2004) as "imperfect knowledge concerning the present or future state of an organism, system, or (sub)population under consideration." The evaluation of uncertainty has in consequence both qualitative, quantitative descriptive, and prognostic aspects.

Describing the variability, e.g., over individuals (esp. age and sex groups), over exposure situations, over time, over regions, as well as over groups with different behavior and susceptibility should reflect existing differences. These differences might reflect varying contact to hazards, different substance intake or the body burden. Variability and heterogeneity refer to the natural variations (IOM 2013) in the environment, exposure paths, and susceptibility of subpopulations. They should be seen as inherent characteristics, which cannot be controlled by the exposure assessor or the decision makers. Variability and heterogeneity cannot be reduced by collecting more information, only by a stepwise selection of more homogeneous subgroups (stratification of analysis) within the evaluation process.

Uncertainty in exposure assessment refers to any lack of knowledge regarding the true value of quantities describing the real or the expected exposure. Any uncertain information used in the exposure estimation process will lower the confidence into the validity of exposure assessment results. The National Research Council (NRC 1994) stated: "Uncertainty forces decision makers to judge how probable it is that risks will be overestimated or underestimated for every member of the exposed population, whereas variability forces them to cope with the certainty that different individuals will be subjected to risks both above and below any reference point one chooses" (NRC 1994, p. 237).

Development of a Regulatory Status of Uncertainty Analysis

Within the last decade, the documentation of uncertainty as a necessary part of any exposure and risk assessment has become mandatory for accepted chemical safety dossiers in the United States of America and Europe (U.S. EPA 2001, 2019; EU 2003; ESFA 2006; ECHA 2008, 2012a, b). Other countries have adopted the approaches (e.g., MEP 2012).

A key document that is used as a framework in all regulatory approaches is the IPCS-WHO (2008) guidance document. The structure of this overview follows the outline and the guiding principles described there. The terminology is mainly in accordance to IPCS/WHO (2004, 2008), specific terms in the context of REACH are described in ECHA (2008). A glossary of terms in the field of food safety is published by EFSA (2012).

Uncertainty analysis plays a central role in risk communication. It might clarify the question which confidence should be given to the risk assessment results in total

and how the reported results might be evaluated in relation to the residual uncertainties. Since the objective of any exposure assessment in a regulatory context is contributing reliable information to the process of decision-making, all sources and all consequences of existing variability, heterogeneity, and uncertainty should be identified (e.g., Morgan and Henrion 1990; Özkaynak et al. 2008). Uncertainty analysis increases the transparency about the state of knowledge, about inherent assumptions, and about the data quality that influences the results of an assessment. The IPCS-WHO (2008) document includes ten recommendations, which summarize methods and some experience with uncertainty analysis.

(IPCS/WHO 1) Uncertainty analysis should be an integral part of exposure assessment.

The recommendation proposes that all steps from the definition of the scope of assessment, the selection of the target variables up to the summary report of the assessment should be evaluated and at least the main results of an uncertainty analysis must be part of the documentation.

Uncertainty analysis should display which information might be assumed to be sufficient reliable and which should be used with caution. Furthermore, an uncertainty analysis might clarify which steps and actions might be taken to reduce the level of uncertainty. A sensitivity analysis as part of uncertainty analysis might additionally contribute important information for the risk management process:

It will clarify which model variables (influence factors) have a high impact on the overall exposure. A comparative evaluation of the costs, the time and the necessary efforts for an increase in quality on one side and the expected information gain for the risk management process on the other side might be an additional result of such an evaluation.

An exposure and risk assessment should be organized as a stepwise process (tiered approach) that starts with a simplified approach, e.g., with simplified scenarios, simple models, and/or with defaults for reasonable upper-bound estimates for all model variables. Such screening approaches should mostly overestimate the real population exposure since it is based on conservative assumptions in terms of influence. However, the approach has the advantage of simplicity, not requiring detailed information about each variable. Using appropriate parameter values the calculation might result in an estimate with high coverage of possible exposure and risk. If no risk is identified with such a screening methodology, it is not necessary to use more sophisticated calculation tools (EFSA 2007). If the documentation of inherent uncertainties does not indicate to restrictions with respect to an interpretation of results, even such a simplified analysis might be useful for the management decision. But such simplified approaches should generate valid upper-bound-estimates of possible exposure for the population under consideration, with a low degree of inherent residual uncertainty.

(IPCS/WHO 2) The level of detail of the uncertainty analysis should be based on a tiered approach and consistent with the overall scope and purpose of the exposure and risk assessment

If the quality assessment points to relevant limitations or if the results indicate to uncertain, but relevant results, an iterative refinement of the scenarios, of the models, and of the data basis will be necessary. Under these circumstances, a refinement would be required to achieve a sufficient quality of the results.

A simplified upper-bound exposure assessment together with an uncertainty analysis might have a high value in risk communication: The management might use the preliminary results as a first and timely, but uncertain, estimate. The risk management might furthermore describe the ongoing and planned steps to clarify the exposure situation, requiring iteration. And, the exposure assessors will have a justification for a time- and resource-binding refinement of the exposure assessment on a higher tier.

(IPCS/WHO 10) Communication of the results of exposure assessment uncertainties to the different stakeholders should reflect the different needs of the audiences in a transparent and understandable manner.

Communicating uncertain information in parallel with a description of the inherent problems, joined by a statement about necessary or ongoing steps to reduce uncertainty, might have a higher degree of perceived accuracy and credibility than waiting for complete information. Giving no or restricted information to the public is communication too. If necessary, even decisions for controlling existing risks might be made on a provisional basis, subject to verification or revision. It is the responsibility of the exposure (and risk) assessment experts to explain the inherent uncertainties. Since the audiences of risk communication may differ with respect to knowledge in the field, interests, and demands, the task of explaining exposure assessment results together with an uncertainty analysis will always be difficult. A detailed analysis of uncertainties will support the risk communication process with respect to the demands, to arising individual questions, and to the general requirements.

Rationale for Characterizing Uncertainty in Exposure Assessment

The evaluation of human health risks requires information about the pollutant (e.g., emission rates, physical and chemical characterization of the substances, rates of degradation, and transformation), environmental concentrations, sources and pathways of exposure, and exposure/dose-response data. Information about each of these assessment elements might be limited. The identification of critical gaps in knowledge (scenarios and models) and data quality will be supported by a stepwise evaluation of uncertainties.

(IPCS/WHO 3) Sources of uncertainty and variability should be systematically identified and evaluated in the exposure assessment

Definition of Assessment Objectives

The assessment objectives should be clearly defined. "Which information is of most interest?" This question has to be decided together with the risk management by the risk assessor prior to any exposure analysis. Within the first phase of an assessment, the reduction of language-based uncertainty should be seen as a communication target. Precision of language is often overlooked as a source of uncertainty in this phase of the assessment (Carey and Burgman 2008), it can result in misunderstanding, lost efforts, and delay. In general, exposure assessment should provide information about the nature of the source(s) and route(s) of exposure as well as information about the individuals who are exposed (Cullen and Frey 1999). Two different purposes for exposure assessment might be distinguished, (a) to assess the safety of legal limits (e.g., preregulatory dietary exposure assessment), or (b) to assess the actual exposure situation of a population or a specific subgroup (post-regulatory exposure assessment).

For regulatory purposes a mayor question that should be answered is "Do the results indicate to exposure higher than a predefined critical limit?" This requires in general a comparison to TDI, ADI, PTWI, or DNEL values. The unit of evaluation, in general expressed as [mg substance per kg body weight for a given time scale (maximal daily, average daily of long-term exposure)] should be defined in advance. If the results of an exposure assessment indicate, even in parts, a "higher/near the evaluation level" answer, (a) those ranges of the input variables that generate high exposure or risk, (b) those subgroups which show high exposure, and/or (c) those specific sources and pathways contributing to this situation should be identified (IPCS/WHO 1994, 2005). This requires qualitative evaluation, quantitative ranking of relevant inputs variables, and a discrimination of the importance among the influence factors. By this, input variables (and their inherent variance) that do not contribute to critical results could be separated from those influence factors (variables) that contribute mostly to high exposure conditions. It is the task of the exposure assessor to clarify the influence structure together with the identification of possible error sources and uncertainties.

Typical questions of the management and the public which call for an uncertainty analysis (Saltelli et al. 2004) are: (a) How confident are you in the results? (b) How much will the results change if the basic (input) data is slightly wrong or will change over time, over regions, over subgroups? (c) Which impact any change of input data and assumptions will have? (d) Which of the uncertain input factors is most important in determining the output? (e) If we could invest in elimination of uncertainty in one of the input factors, which factor should we start with to reduce the uncertainty of results?

Once the objectives are defined, the assessment must be designed to address them. An emerging challenge is how to quantify variability and uncertainty in integrated assessments over the source to exposure to uptake continuum. Since in general many scientific fields are tangled, any exposure assessment process should be seen as an interdisciplinary approach.

Sources of Uncertainty in Exposure Assessment

The IPCS-WHO (2008) harmonization document calls for an analysis and full description for characterizing uncertainty using qualitative as well as quantitative approaches. Although inconsistencies in the application and methodology of uncertainty analysis might be seen, comparing the recommendations of different organizations, some common elements should be highlighted – these include qualitative and quantitative approaches.

(IPCS/WHO 7) Uncertainty analyses for exposure assessment should be documented fully and systematically in a transparent manner, including both qualitative and quantitative aspects pertaining to data, methods, scenarios, inputs, models, outputs, sensitivity analysis and interpretation of results.

The level of uncertainty that is contributed by the selection of scenarios, the conceptual and mathematical model applied and the choice of parameters should be documented. A qualitative evaluation should include the appraisal of the current scientific knowledge base. Controversial sources of uncertainty should be referred to and a (qualitative) evaluation of inherent subjectivity of choices for each of the controversial sources should be presented.

(IPCS/WHO 5) Data, expert judgement, or both should be used to inform the specification of uncertainties for scenarios, models and model parameters.

If different scientific approaches are available, then evidence and plausibility, the scientific support and the consistency of methods and data should be considered. The robustness of results using different assumptions and models (choice space) should be checked. By this, a full uncertainty analysis might offer a framework to facilitate and promote a qualitative consideration of the impact that uncertainties might have on the exposure assessment's results.

Scenario Uncertainty

The scenarios should describe how people may be exposed to substances by emission, by ambient air pollutants, during manufacture, during industrial, professional, and consumer use of products as well as during the service life of articles and products. In principle, all scenarios do not reflect one specific local situation, but have the objective to be representative of either mean, typical, or most sensitive situations in a region for a defined population (EC 2000). Scenario uncertainty includes possible descriptive errors (e.g., wrong or incomplete information about the facts), aggregation errors (e.g., approximations for population subgroups, time scale, season, and regional differences), errors of assessment (e.g., choice of provisional model, extrapolation from other exposure situations), and errors of incomplete analysis (e.g., overlooking/ ignoring important exposure pathways and sources).

For exposure to chemical substances, ECHA (2008) proposed some rules for considering exposure scenarios: If the intended use of a chemical is known, as it is assumed in ECHA regulations, then a detailed description of all resulting exposure scenarios is required. The type and the number of exposure scenarios depend on how the substance is used in a predicable manner. Attributes that trigger the description of exposure scenarios are the sectors of use (SU), the product category (PC), the article category (AC) together with the environmental release category (ERC). For exposure in occupational settings the process category (PROC) should characterize production- and application-related characteristics. For consumer exposure, the product categories are defined in ECHA's Guidance R.12 (2008), describing the scope of exposure scenarios.

Uncertainties might arise, (a) if the identified uses are not consistent with other sources of information, if (b) identified uses are not covered by exposure scenarios or, (c) if operational conditions do not seem to be sufficiently realistic. Within a REACH chemical safety report (CSR) the description of all exposure scenarios should ensure the safe use of the substance. The necessary control measures must be described by the manufacturer or the importer. All determinants that reflect the conditions of use and the risk management measures should be reported within the exposure scenario description. Model assumptions should be reported in the exposure tables included in the chemical safety reports (CSR).

The variability in consumer behavior and the recognition of possible multiple exposures to the same substances from different products have to be taken into account in the consumer exposure setting. Additional information about the scenario description and the assessment methods are available in the ECHA (2010, 2011, 2012b) guidance documents which includes several practical examples.

A concise description of exposure scenarios might be used as a starting point for a conceptual description how exposure might occur. Relevant exposure events might differ over age (e.g., due to behavior, consumption, sources), sex/gender (e.g., with respect to behavior like using cosmetics, product usage), and region (e.g., by nutritional habits and environmental conditions).

A valid and reliable estimation of exposure requires appropriate description of scenarios, scientific concepts to translate this knowledge in to appropriate models and adequate formulas within a mathematical model to represent the exposure scenario.

Model Uncertainty

Any mathematical model corresponding to an exposure scenario should reflect the dependencies of the degree of exposure in relation to all influential factors. The identification and description of all relevant exposure scenarios is an important prerequisite. An exposure assessment should provide full information about the origin of the model together with a detailed description and its validation status. This includes all formula(s) and a brief description of all variables. The set of involved variables needs a definition with respect to the content and the units

used. A list of all parameter values or distributions that represent the exposure factors within the population under concern should be part of the documentation. Any uncertainty that is related to the exposure scenarios will propagate to the exposure model and will influence the uncertainty of results. A general structure of exposure models includes information for each route/pathway (oral, dermal, inhalation) as well as for all exposure sources: the contact or intake frequency, the amount of transfer per contact/intake/uptake, as well as information about the concentration of the substance per item unit (e.g., mg Me Hg/kg Fish fresh weight, μg NO2/m^3 air). The total exposure is calculated as the sum over all pathways each including a sum over all contributing sources. The specific time intervals for all these intake-related variables should be defined in a homogeneous way.

$$\frac{Intake}{Time} = \sum_{i=1}^{Path} \times \left(\sum_{j=1}^{Sources} \frac{ContactFrquency_{Sources}}{Time} * Intake_{Event/Time} * Concentration_{Sources} \right) * Transfer_{Path}$$

Although the structure of the model is simple, the collection of information for an exposure model with many sources for exposure and different contributing pathways is demanding. For each pathway, at least one variable for a transfer factor is necessary. If the concentration of the substance of interest is changing in preparation (e.g., peeling or cooking/frying) or if concentration data is only available for whole food concentration then a transfer factor should describe the corresponding rate of change in concentration. If the internal (ingested/absorbed) dose is the target variable, assessing the dose within the body after the agent enters the body via ingestion, inhalation, or dermal absorption requires a transfer factor including sufficient information about the rate of absorption. The intake by each pathway (oral, dermal, inhalation) is a sum over all contact items (sources). All sources of exposure (e.g., food items, contact material, product application, indoor and outdoor immission) must be considered, as long as not at least one of the multiplication terms of the exposure equation equals (near) zero.

It should be noted that variance, measurement errors, and uncertainty of each element in the calculation propagate in a factorial manner (multiplication). The uncertainty of each source-related exposure is dependent on the quality of information of all elements in the part of model equation. The errors ε_i of each source-related intake estimate, describing the total of uncertainty for this item (e.g., the average methylmercury intake MeHg per day by tuna consumption) will increase the total error in a multiplicative manner.

The measured (or estimated) value of each parameter might be described as a composite of the true value x_i and an error ε_i, the latter dependent on the uncertainty of each variable (e.g., for the estimate of methylmercury intake by tuna the frequency of consumption, the amount eaten per meal, the MeHg concentration in fresh tuna,

and transfer factor for preparation factor and absorption rate). The type of error linkage might be additive ($V_i = x_i + \varepsilon_i$) or multiplicative ($V_i = x_i * \varepsilon_i$), depending on the variable.

$$\textbf{Total Error of intake estimate}_{\textbf{Item i}} = \varepsilon_i \sim \varepsilon_{\textbf{frequency}} * \varepsilon_{\textbf{amount}} * \varepsilon_{\textbf{concentration}} * \varepsilon_{\textbf{transfer}}$$

The error of the intake estimates of each source is the multiplicative combination of all errors. Any systematic shift or error in exposure frequency, of the amount consumed, or in substance concentration will result in an error of the exposure estimate. The sum of substance intake over all items (e.g., methylmercury exposure sources) per pathway might include many partial calculations (e.g., with varying consumption of different fish/food species with varying substance concentrations). Each might have a different quality. An exposure assessment integrates all the information about the sources and the relevant pathways into one exposure estimate. In consequence, the uncertainty analysis gains complexity. At least a basic evaluation of possible error sources is necessary to avoid wrong or distorted estimates.

The lack of quality might be a result of the model selection too. Describing an average exposure (per day, per week, per month) will require statistical information about average contacts, average frequencies, and average amounts of use, consumption, ingestion, or inhalation together with information about the substance concentration over time. A model that is describing exposure in an event-based manner requires much more information (e.g., the number of hand-to-mouth-contacts for toddlers per time unit, the contamination distribution of the contact environment over a certain period, the substance transfer by hand-to-surface-contact and by hand-to-mouth-transfer). In consequence, the time scale of the model variables and the information about the variables should be in accordance with the time scale of the target variable of the assessment.

Exposure models might describe different periods of time: The temporal scale for estimating exposure (and dose) depends on the scope of assessment. These might be peak doses (aRfD: acute reference dose), exposures occurring over a very short period of time (e.g., minutes), time-weighted averages, or exposure per day (e.g., for ADI, TDI, RfD comparison) or doses per week (e.g., for PTWI comparison). Exposure models should express the total intake for a specific time interval as the sum over all relevant pathways: e.g., dietary and nondietary intake (oral), skin contact (dermal), and inhalation.

$$\text{Intake}_{\text{total exposure}} = \text{Intake}_{\text{oral}} + \text{Intake}_{\text{dermal}} + \text{Intake}_{\text{inhalation}}$$

The errors and uncertainties of the path related intake estimates $\varepsilon_{\text{total exposure}}$ will add up over all pathways. In general, the contribution of each path to the total exposure should be documented. An evaluation of inherent uncertainty per pathway is recommended.

$$\text{Error of estimate}_{\text{total exposure}} = \varepsilon_{\text{total exposure}} = \varepsilon_{\text{oral}} + \varepsilon_{\text{dermal}} + \varepsilon_{\text{inhalation}}$$

The magnitude of exposure is in general reported as an approximation of a risk-related numerical value, the total exposure divided by the body weight (as a proxy for the distribution volume). By this, the exposure estimate and the regulatory values, e.g., for the TDI, ADI, PTWI are reported in unified units [e.g., mg substance/kg body weight per time unit]. The step of dividing exposure by body weight introduces some additional uncertainty: (a) body weights show variation, (b) the intake (e.g., water, and food consumption) might be correlated to the body weight, (c) the relation between intake (e.g., breathing volume) and age might show non-linearities and (d) the relationship between nominator (exposure) and denominator (body weight and time scale used) might be moderated by other influential factors (e.g., level of activity, cultural and nutrition habits). All these relations might result in a lack of independence of the parameters. If these influences might result in systematic over- or underestimation, correlation and dependency between variables of the model must be included into the assessment.

(IPCS/WHO 4) The presence or absence of moderate to strong dependencies between model inputs is to be discussed and appropriately accounted for in the analysis.

Good modelling approaches use sensitivity analysis as a companion tool to identify possible errors (e.g., by evaluation of predictions of the model results against known data as a model calibration). Sensitivity analysis might demonstrate the possible impact of dependencies (e.g., described by correlation between the input variables).

Parameter Uncertainty

As a starting point for a (deterministic) exposure assessment in general, default values (single-value-estimates) are used. These defaults should correspond to a description of the central tendency (mean, median of the parameter distribution representing the target population) or should stand for an upper-bound-estimate (e.g., reasonable-most-exposed (RME) in general described by 95%-distribution coverage of the particular variable. If the assessors intended as a screening step to produce conservative estimates of exposure (Hart et al. 2002), a combination of RME values for variables in the nominator (e.g., consumption per day, concentration) and lower-bound-estimates (e.g., 5%-quantiles) of the denominator (e.g., body weight) should be used for calculation. It has to be provided that the choice of a model and RME default parameters include appropriate conservative assumptions to take account for uncertainty. The Scientific Committee of the EFSA (2006, 2007) recommends that each scientific panel should review whether this requirement is satisfied by the assumptions and default values that they used previously. Treating the most significant uncertainties at each refinement step (higher tiers) progressively should refine the characterization of uncertainty about the likelihood of exceeding health-based guidance values. This should be done stepwise by evaluating the variability and the uncertainty in an integrated assessment. The numerical

description of uncertainty in parameters might be given as (a) symmetric confidence intervals (e.g., defined by standard deviations), (b) defined quantile ranges and error bands, or (c) as asymmetric confidence bands [$CI_{lower\ bound}$, $CI_{upper\ bound}$] for skewed distributions. A short justification for each selection should be given.

Uncertainty in Measurement

Ideally, any measurement informing an exposure assessment would be free of random error and should not be influenced by systematic error. The higher the quality of a measurement instrument with respect to accuracy (bias) and precision, the lower the uncertainty will be. Random error is associated with the fact that repeated measurement in general will provide different measured values although the attributes of the object are assumed to be constant over time. The term "random error" describes the unpredictability of the deviances in a series of measures. Random error of a model parameter restricts the reliability. If a numerical estimate of the random error is available (e.g., by repeated measurement→reliability), the quantitative impact of random errors on the exposure results might be evaluated directly.

Systematic errors generate shifts on the measurement scale of model parameters. They might depend on external influence factors (e.g., differences over measurement instruments, over observers, over laboratory standards, and in relation to conditions of measurement and sampling). The degree of confidence about the absence of systematic error is described in general in a qualitative manner. If the direction of a systematic error is known, but not its magnitude, then the impact on the results might be estimated only in a qualitative manner. If a systematic error might be described by numerical boundaries, then the range of a possible quantitative impact on the results might be estimated too.

The resulting one-dimensional uncertainty interval of the results might describe the range of "true" value(s) of the outcome. For a detailed description and discussion of dealing with uncertainty in measurement, we refer to references for standards of measurement (e.g., ISO 1993; ASME 2005; JCGM 2008; NIST 2011).

Exposure assessment involves the specification of numerical values for all variables which are included in the exposure model. Selecting appropriate parameters for the model's variables is a crucial factor for the model validity. But, with few exceptions the data available for an assessment will not be closely related to the exposure scenario (e.g., specific subgroups of consumers, regions) that has given rise to the request from the risk managers. In consequence, there will be always uncertainties, most of which cannot be quantified (EC 2000, p. 38) but discussed in a qualitative manner.

Data Sources for Model Parameters (Exposure Factors)

Numerical default values for exposure parameters are obtained using various approaches (e.g., expert judgement, statistical analysis) and different sources (e.g., survey data, consumer panels, market observation). Within the last years, several countries have reported National Exposure Factor handbooks. Those

collections with a longer tradition back to the 90th are U.S. EPA's Exposure Factors Handbook (for adults: U.S. EPA 2011, for children: 2008, 2009), the European Union's (EU) Technical Guidance Document (EU 2003), the German XProb project (AUH 1995; Mekel et al. 2007, UBA 2011), and the European KTL's ExpoFacts (Vuori et al. 2006; JRC 2010) and the ConsExpo Fact sheets (RIVM 2012a, b).

Adjacent to reporting default values (e.g., median, mean, upper quantiles), these documents include information about the parameters: (a) descriptive statistics including variability, (b) the cumulative distribution and in parts (c), the type of underlying distribution (EPA 1999). In general, a stratification for age and sex, and if necessary due to population heterogeneity, stratification by ethnic groups is included. "Variability and heterogeneity refer to the natural variations in the environment, exposure paths, and susceptibility of subpopulations. They are inherent characteristics of a system under study, cannot be controlled by decision makers, and cannot be reduced by collecting more information." (IOM 2013, p. 3). Statistical uncertainty of estimates resulting from restricted sample size, are in parts reported for single-value-estimates (defaults). By this, conducting statistical uncertainty analysis using default values and confidence intervals is possible (see, e.g., Filipsson et al. 2011). Uncertainty due to sampling strategies (e.g., selection of study participants, response rates, regional differentiation) require a qualitative evaluation. Using the exposure factors (and variability indicators) published on a national level will result in general in an accepted state-of-the-art exposure assessment.

Uncertainties inherent in parameter values for exposure factors can be classified as sampling and non-sampling errors. Sampling errors arise from limited sample sizes in relation to the population size under consideration. The magnitude of this error is a function of (a) the variability of the measured attribute and (b) the sample size. In practice, we have to deal with the situation, that we have very low sample sizes, mostly for contamination measurement (esp. for food items, environmental measures). In these cases, the confidence intervals of the mean, the median and much more those for the upper quantiles show wide ranges. The selection of a type of distribution, in these situations, is based merely on scientific experience, not on data. If we only have data from selected subpopulations, we must assess the degree of representativeness for the target population and the expected effects of deviation. For selected scenarios and for statistical uncertainty calculation examples see e.g., Hammonds et al. (1994), IAEA (1989), RIVM (2009), U.S.EPA (2008, 2011).

More general problems might occur if exposure magnitude should be estimated for specific periods of the life span (child development, pregnancy, occupation). The age stratification of exposure factor handbooks is restricted. Especially for developmental studies, any changes in the exposure media, with respect to the sources and the pathways over the life stage should be considered. Each developmental stage requires the selection of specific scenarios, models, and appropriate age-related parameters – and a specific uncertainty evaluation (U.S. EPA 2006a).

Evaluating the Total Impact of Uncertainty

The objective of a full characterization of uncertainty of an exposure assessment includes transparency, the identification of key sources of uncertainty and an evaluation of the consequences of limited information in the decision making. A systematic qualitative characterization of the sources of uncertainty is encouraged, as it provides the appropriate degree of confidence in outcome and associated recommendations. Short overviews of concepts of and methods that might be useful for reading assessments and for the evaluation in parallel to preparing an exposure assessment are given in IPCS-WHO (2008), EFSA (2006), ECHA (2006), and BfR 2015.

A simple documentation scheme for identified uncertainties (Table 1) is proposed by IPCS-WHO (2008). The rows reflect the steps of exposure assessment, the column headers might be used as a guide for the identification of the mayor sources of uncertainty: Each element of the matrix contains many aspects that might contribute to the overall uncertainty of exposure assessment results. For each element a classification of the uncertainty should be assigned. IPCS-WHO (2008) recommends the terms (No, Medium, High or NA = "not applicable") for the quality and uncertainty assignments; EFSA (2006) proposed a ranking using two "+" and "−" signs indicating the direction and the magnitude of uncertainty for each subject of consideration. Short verbal descriptions of relevant uncertainty aspects for each cell of the table will support the transparency of the documentation.

Model Evaluation

The promise given by an exposure assessment is, that the estimated results would approximately reflect the real exposure situation for a defined population. According to the classification of exposure assessment methods, data and model-based exposure assessment belongs to the class of indirect measurement. They utilize existing

Table 1 Modified version of the EFSA (2006) and IPCS-WHO (2008) evaluation scheme

	Characteristics of uncertainty		
Sources of uncertainty	Overall level of uncertainty	Appraisal of the knowledge base	Subjectivity of choices
Scope/assessment objectives			
Scenarios			
Conceptual model			
Mathematical model			
Parameters			
Result(s)			

(secondary) data on chemical concentration, frequency, strength, and duration of contact, without doing any specific measurement of the outcome variable.

In contrast, a point-of-contact approach involves measurements of chemical concentrations at the point where exposure occurs to assess the outcome variable. These quantify concentration close to the interface between the person and the environment (e.g., by personal samplers, by personal protocols, or duplicates of dietary intake). If the time interval of contact is recorded, the average exposure per time unit might be calculated. This type of exposure estimate requires data from environmental samplers (e.g., measuring pollutants in indoor or outdoor), information of the individual's characteristics (e.g., breathing rates), time-budget in different environmental media like indoors, outdoors, in cars. An example from Payne-Sturges et al. (2004) shows, for instance, that personal sampler-based exposures measures show higher values than exposure calculations for indoor volatile organic compounds (VOC) exposure based on standard exposure factors. Personal monitoring might reflect the variance of exposure conditions better than exposure estimation.

Since the target variables of an exposure assessment should reflect the uptake of a substance in relation to the distribution volume (indicator: body weight), the most appropriate information for comparison exposure estimates stems from biomonitoring studies. For example, Xue et al. (2010) studied the intake of inorganic arsenic in the general US population with the objective to compare exposure model predictions with observed biomonitoring data (see also NHANES 2020). The goal was to quantify the distribution of total dietary arsenic exposure. Comparing model predictions with observed data, the evaluation was conducted via comparing exposure and dose-modeling predictions against duplicate diet data and biomarker measurements, respectively, for the same individuals. The distribution of the modeled exposure (biomonitoring with pharmacokinetic dose estimation) and the distribution of estimates of exposure matched well with the distribution of the Duplicate Diet estimates. Kurzius-Spencer et al. (2013) show that Total Diet Studies (TDS) might underestimate the dietary intake in comparison (a) to modeled dietary arsenic exposure based on 24-h duplicate diet samples intake, (b) exposure estimation using distributions as well as compared (c) to backward estimation of arsenic intake from 24-h urine measurement.

The use of biomarkers of exposure may provide a more detailed and less biased estimate of substance uptake and distribution than any indirect methods. But this requires full information about the distribution in the body and metabolism of the substance. The linkage of biomonitoring data to specific sources requires again exposure models (U.S. EPA 2006b). Burns et al. (2014) describe the sources of uncertainty associated with the results of most epidemiologic studies together with techniques that exist that can be applied to improve weight-of-evidence evaluations and risk characterization efforts. Only few evaluation studies have analyzed the predictive quality of exposure assessment for Human Biomonitoring data in detail. In consequence, the usage of exposure assessment should be directed to a comparison of prognostic results to regulatory recommendations for substance intake (like ADI, TDI, RfD, DNEL).

Sensitivity Analysis

Cullen and Frey (1999) define sensitivity analysis as the assessment of the impact of changes in input values on model outputs. Sensitivity analysis is used to determine how different values of an input, the independent variables, will impact a particular output, the dependent) variables, under a given set of assumptions. Sensitivity analysis studies the relationships between information flowing in and out of the model (Saltelli et al. 2004). Frey and Patil (2002) underline the use of sensitivity analysis in exposure assessment for an identification and comparison of sources of uncertainty that influence the target variables and the assessment conclusions. Furthermore, a sensitivity analysis is useful for providing insight regarding model verification and the robustness of models (Cullen and Frey 1999).

WHO/IPCS 6) Sensitivity analysis should be an integral component of the uncertainty analysis in order to identify key sources of variability, uncertainty or both and to aid in iterative refinement of the exposure model.

If risk managers like to consider the impacts of alternative regulatory or risk management choices than sensitivity analysis is inevitable. Any maximizing of benefits will depend on a comparison of results based on scenarios, models, and parameter alternatives. Incorporating variability and uncertainty into such comparative assessment is state-of-the art. Ignoring variability would mean neglecting existing differences in exposure conditions (e.g., over persons, over exposing situations, application/usage/contact of hazards). Ignoring uncertainty would mean providing results as reliable, even if they are questionable.

The identification of model variables which are not controllable by the risk management (e.g., breathing rates, body surface area, body weight) will inform about the limiting conditions that might not be changed by regulation, control or advice.

Uncertainty concerning causal analyses must be characterized qualitatively. A qualitative judgment of the overall uncertainty should be accompanied by a list of major sources of uncertainty and a quantification of the expected influence of variation of the parameters on the results. Variables that might not be modified can have a high impact on the outcome (e.g., breathing rates, water consumption). Using for instance physiologically based pharmacokinetic (PBPK) models to predict the dose of a chemical substance or metabolite will result in a strong dependency of many model parameters to organ weights or body weight (see, e.g., Farrar et al. 1989; Krewski et al. 1995; Clewell et al. 2000; U.S. EPA 2006b; Bois et al. 2010). The identification of all variables that have a high influence on the target variable requires quantitative analysis.

Building a ranked list that describes the influence of the input variables on the target variables requires statistical analysis. The goal is to quantify the degree of influence of the input variables variance on the variance of the target variable. An analysis of all possible outcomes for all ranges of the input variables (variability), together with a consideration of inherent quantitative (and numerical expressed

qualitative) uncertainties, is a scientific task that will call for an involvement of mathematical, statistical and exposure science expertise. This has to be considered if the models include many pathways and sources. If the global exposure model contains several submodels for influence factors, uncertainty evaluation should be conducted by scientists from different faculties.

Quantitative Sensitivity Analysis: Identification of Key Sources of Exposure, Uncertainty, and Variability

Since the efforts for a statistical sensitivity analysis should be balanced with respect to cost and time versus the expected gain of information, any uncertainty analysis should start with a screening step, which uses defaults for all parameters, evaluating the change of the outcome by stepwise changing these values. The identification of those input variables that have a strong influence on the variance of the target variables will indicate to variables with a high potential for possible exposure control (Frey et al. 2004).

The impact of variability might be controlled by a parameter-wise alternation of central tendency default to an upper-bound-estimate (e.g., the 95%- or 5%-quantiles). This procedure gives an overview about the 95% ranges of an influential variable, keeping all other influence variables on the mean or median (default for central tendency). In a similar manner, the impact of statistical uncertainty might be controlled by a parameter-wise alternation of the central tendency and/or the upper-bound-estimates using the confidence intervals (a fixed percentage) of these values, this describes the degree of uncertainty due to statistical reasons about the stability of estimates. These "One-At-A-Time" (OAT) methods (Murphy et al. 2004), changing always only one input parameter while keeping all other values constant, are strongly recommended at the screening level. A "tornado diagram," ranking the variables by the outcome change, might illustrate the relative importance of each input variable. This approach corresponds to the economic evaluation term "elasticity" that describes sensitivity as the ratio of the percentage change in one outcome variable to the percentage change in an input variable; it is in general calculated as the ratio of changes in natural logarithmic units: $E_{x,y} = \ln(\text{change in output y})/\ln(\text{change in input x})$ which corresponds approximately to (% output change in y)/(% input change in x). For an overview of graphical methods for presenting quantitative uncertainty, see, e.g., Ibrekk and Morgan (1987) and Edwards et al. (2012).

The main advantage of these One-At-A-time approaches is the fact, that the resulting changes in the model outcome are directly related to the change of input. These methods are simplified approaches for gaining information about the slope of change (mathematically the local partial derivate) at a given point in a multidimensional problem. An evaluation of results based on a One-At-A-Time approach is in general understandable for risk managers and the public. But it describes only the effects of variability or uncertainty for selected values, possible interactions between variables and nonlinearity are ignored. The behavior of the model might deviate if all variables show variation (and uncertainty) in a multivariate setting with dependencies and interaction (Cacuci 2003; Murphy et al. 2004).

Taking into account the combined effects of many input variables requires data sampling plans similar to experiments (e.g., Morris 1991) or simulation analysis. Looking at the variance impact is mostly done by Monte Carlo (MC) simulation. If the uncertainty of the parameters might only be described by ranges (numerical intervals), then these values might be used too (e.g., in a uniform distribution). If relevant association exists between the input variables, information about the correlation (covariance) structure should be used in the simulation model. Technically a Monte Carlo simulation (see, e.g., Fishman 1996) consists of random combinations of random variates following the distribution of each input variable. Repeating these random choices many times, the distribution of the exposure variable will represent the set of all possible combination of input variables, constraint by the distribution of input. Technically, these approaches are called probabilistic. If uncertainty is included into the simulation, the analysis changes from a one-dimensional variance propagation model into a two-dimensional analysis. WHO-tools that combine the output from the probabilistic hazard characterization with the probabilistic exposure to rapidly characterize risk and its uncertainty are described by Bokkers et al. (2017).

Using Monte Carlo simulations, the dependency of the output values on the input variability might be evaluated by a variance-based approaches. Typical methods are: (a) drawing scattergrams for visual inspection of dependency, (b) calculation of rank correlation calculation describing the ordinal degree of the "the more/less of input, the more/less of exposure). Ranking the correlation coefficients of the variables by the degree of association gives information that illustrates the positive and negative impact of the input variance and the degree uncertainty on the calculated exposure variance. (c) Calculation of a regression models with the input variables as independent and the exposure estimate as the dependent variable allows an integrative view. Using the standardized regression coefficients, allows a direct comparison (Cacuci 2003).

Introducing quantitative estimates of uncertainty into a variation-based model results in a calculation that consists of a (in general additive) mixture of variation and uncertainty. Uncertainty and variance compounds need to be represented by different variables within the model. They should be used as different terms in the (rank) correlation and regression calculations too. The combined effect might be evaluated. But this approach presumes a lot of information about the set of variables. In practice, a full sensitivity analysis including variability and uncertainty components is rarely done. If necessary due to model complexity or safety requirements, even more elaborated mathematical methods (e.g., Saltelli et al. 2004) might be appropriate.

If raw data sets from representative samples of the population (e.g., collected as national surveys) are available, then the original data set might be used as a calculation basis for exposure estimation. Using the individual consumption frequencies, the individual amounts eaten/used together with the individual anthropometric data (e.g., body weights), only the substance concentration distribution needs to be simulated according to the information about the type of distribution. The calculation results in a population-based estimate of exposure. This approach avoids the problems of data dependency, correlation, and interaction and reduces data and

model uncertainty. The techniques for a sensitivity analysis are the same as described above.

An approach of stratifying for homogeneous subgroups (e.g., age, sex, region, nutritional habits) will reduce the variability within each stratum (subgroup) but will keep the variation over the groups. Stratification rules should be guided by attributes that are reasonable linked to exposure. Differences in behavior (e.g., typical activities, consumption habits, product usage) might provide an indication for such a classification. This might be done by an exclusion of the exposure sources. Alternatively, the scope of the assessment might be changed, e.g., developing a model tailored for the "exposed fish-eater group" (see, e.g., EFSA 2012b).

If a sensitivity analysis identifies uncertainties in relation to knowledge about important data, this should be seen as a prioritizing argument for additional data collection or research. By this, it justifies a higher tier analysis and further iteration (Recommendation 2 of the IPCS-WHO 2008).

Interpretation of Uncertainty Characterization Results

Exposure assessment is based on scenarios, models, as wells as sufficient data about all influential exposure factors. The result of an exposure assessment is a prognosis about the expected level of exposure or the resulting body burden. Instead, direct methods of exposure assessment, such as personal sampling, duplicate studies, and human biomonitoring provide information on a measurement level. In consequence, exposure assessors and risk managers should balance the reasons for using prognostic techniques instead of direct exposure measurement methods. The main advantage of using exposure models over direct measurement is cost and time – in general at the price of a higher degree of uncertainty.

A prerequisite for exposure analysis is that the state of knowledge about all the different influence factors is sufficient and that existing knowledge might be translated into an exposure model. The assessor should keep in mind, why an assessment was required, which problems and which questions have trigged the request. Zehr (1999) pointed to the problem that "... unknowns, indeterminacy and ignorance, that exist in science are often transformed into uncertainty when reported to the public in order to make issues more manageable." A full and concise uncertainty assessment avoids this, it describes what is known and certain and what might be known doing additional research. Critical questions about the validity of the exposure assessment (accuracy, precision of prediction, validity, and objectivity) that should be expected in the course of risk communication can be anticipated and answered within an uncertainty analysis.

(IPCS/WHO 8) The uncertainty analysis should be subject to an evaluation process that may include peer review, model comparison, quality assurance or comparison with relevant data or independent observations.

The guiding principle eight of the IPCS-WHO document (2008) is related mainly to the questions if the exposure assessment is valid in the sense of scientific sound

quality and if it provides answers that are resistant to critical questions. Identification of uncertainty does not restrict the quality of the assessment. Although it might restrict the utility of an exposure assessment for regulatory or prevention-directed purposes, an uncertainty analysis increases the quality of information. A documentation of information about what is known, what is reasonable to expect, and what needs further clarification might have a high impact on the risk management process.

(IPCS/WHO 9) Where appropriate to an assessment objective, exposure assessments should be iteratively refined over time to incorporate new data, information and methods to better characterize uncertainty and variability.

Where the level of uncertainty is too high, only doing additional research, collecting more information, and/or obtaining better exposure measurements will change the situation.

Cross-References

- ▶ Assessment of Background Exposure and Additional Exposure by Human Biomonitoring
- ▶ Exposure Scenarios in Toxicology
- ▶ Human Biomonitoring: Its Importance in Toxicological Regulation
- ▶ Importance of Exposure Level for Toxicological Risk Assessment
- ▶ Limit Values and Guideline Values in Regulatory Toxicology

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Part IV

Paradigms Used in Risk Evaluation

Read-Across Methodology in Toxicological Risk Assessment

35

S. E. Escher and A. Bitsch

Contents

Abstract

Grouping approaches like read-across (RAx) are one of the most widely used methods to fill data gaps in human risk assessment. In a RAx approach, in vivo animal data from one to several source substances are extrapolated to a target substance that has not been tested. Here, we describe the currently accepted read-across workflow, which begins with the problem formulation that defines the level of the acceptable uncertainty. The evaluation progresses iteratively from an initial list of structurally similar substances to source compounds with similar toxicodynamic and toxicokinetic properties. Finally, the data gap is closed with a worst-case or a regression analysis, and the uncertainty of the prediction is identified. New approach methodologies, such as in vitro assays and in silico models, have great potential to strengthen read-across assessments by providing mechanistic data and estimates of bioavailability of the grouped compounds.

S. E. Escher (✉) · A. Bitsch
Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM), Hannover, Germany
e-mail: sylvia.escher@item.fraunhofer.de; annette.bitsch@item.fraunhofer.de

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Keywords

Read-across · Data gap filling · Human risk assessment · Grouping approaches · Chemical similarity · Biological similarity · New approach methodologies

Introduction

Chemical risk assessment aims at compound-specific threshold values, like acceptable daily intake values, below which an exposure to a compound is considered to be safe for the human population. Within this approach, the hazard assessment provides information on the substance-inherent toxicological properties. This means in a qualitative manner the identification of the type of toxicity caused by the compound of concern and classifying it, e.g., as potentially genotoxic, or reprotoxic, or harmful after chronic lifetime exposure. This information is traditionally derived from in vivo animal studies. In addition, for a quantitative evaluation, a dose level is identified, below which the animals did not show any adverse effect. This dose level is called a No Observed Adverse Effect Level (NOAEL). The NOAEL is thereafter extrapolated to a human threshold value by using appropriate assessment factors that account, i.e., for inter and intraspecies differences (ECETOC 2003).

The absence of appropriate and relevant in vivo animal data is a frequently occurring problem in human risk assessment specially, i.e., in case of an unintended release of compounds to environmental compartments like drinking water or air or in the case of impurities occurring within a production process. This is also of particular importance for the assessment of cosmetics, for which de novo animal testing has been prohibited in EUROPE since 2013 (EU Regulation 1223/2009). Nonetheless, safe levels for human exposure need to be defined. To date, three **alternative non-testing models** can be applied, namely, QSAR models, grouping approaches, and the Threshold of Toxicological Concern (TTC) concept. TTC concept.

Already starting with the OECD HPV program (OECD 2004), grouping approaches have been developed and applied over the last decades as important risk assessment tools. In EUROPE, this development was supported by the REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) legislation, which requires the use of alternative approaches before animal testing is carried out as a last resort. Also, other international agencies like the US EPA and Health Canada apply grouping approaches like read-across for regulatory purposes (Rovida et al. 2020; Patlewicz et al. 2019; US EPA 2010).

Under REACH, grouping approaches like **read-across** (RAx) are the most often applied alternative methods for higher tier endpoints like repeated dose or reprotoxicity (ECHA 2014). A read-across assessment is using the technique of predicting toxicity data for one compound, named as target compound (TC), by using data for the same endpoint from one to several source compounds. The analogue approach uses one, the category approach several source substances for extrapolation (Fig. 1). A read-across assessment is used for hazard assessment and

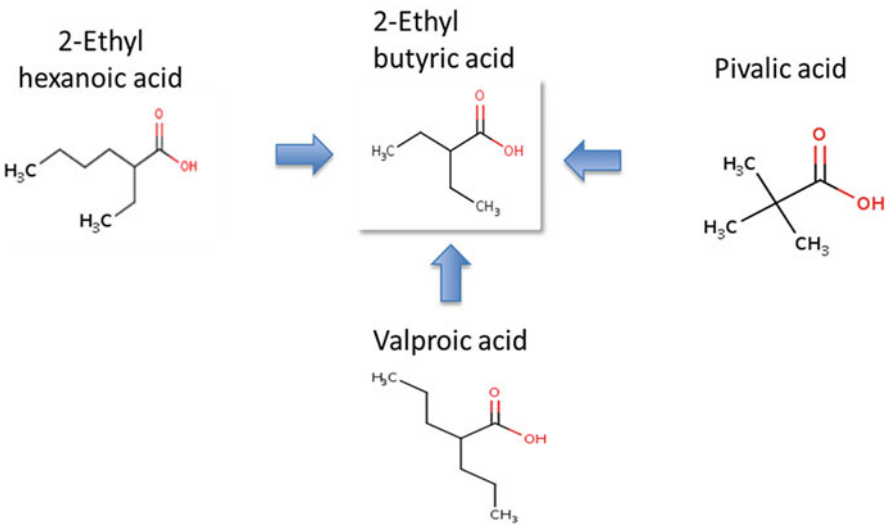

Fig. 1 Illustration of the concept of a read-across evaluation. The toxicity of the target compound "2-ethylbutyric acid" is predicted from the toxicity data of similar source compounds (in this case other branched carboxylic acids), for which appropriate in vivo data are available. A many-to-one (N to 1) prediction is termed category approach, a one-to-one prediction analogue approach (Escher et al. 2021)

can derive qualitative properties, like "is genotoxic," or quantitative estimates, like a NOAEL value.

The authorization procedures under REACH have shown that many of the submitted read-across approaches were not acceptable to ECHA (Ball et al. 2016). The reasons for rejections within the compliance check were manifold, such as (i) lack of sufficient in vivo information from source compounds, (ii) insufficient scientific plausibility, (iii) identity of the target compound not clearly defined like UVCBs (unknown or variable composition, complex reaction products, or biological materials), etc. The low level of acceptance led to the development of the read-across assessment framework (RAAF), in which ECHA outlines in a systematic way the read-across assessment elements (ECHA 2017). A read-across (RAx) case according to ECHA always includes structural similarity. The RAAF distinguishes six different scenarios, which are defined based on the number of source compounds (analogue/category approach) and the so-called RAx hypothesis. This hypothesis must be based on a relationship between structural similarity and the predicted properties and needs to be supported by read-across justification. The RAx hypothesis has to outline whether the toxic effect to be read across is caused by (i) one common toxicant, i.e., formed through metabolism from both the target and source chemicals, or (ii) structurally slightly different source and target chemicals, which cause similar toxicological effects. The latter is also acceptable, if these similar effects follow a trend within the grouped compounds. The RAAF intends to increase the transparency of the assessments and to provide guidance to the applicant on which

assessment elements have to be included in the read-across assessment to be accepted by ECHA. The RAAF, however, doesn't reflect the iterative process required to build a category/analogue approach.

Read-Across: Step-by-Step

The workflow of the read-across assessment comprises six main steps (Helman et al. 2019; Escher et al. 2019, Fig. 2).

From Problem Formulation to the Initial Read-Across Hypothesis

The problem formulation **(step 1)** defines the objective of the read-across and thus the degree of acceptable uncertainty for the obtained extrapolation. For example, if the RAx analysis aims to fill a data gap for a regulatory study, a lower uncertainty is acceptable compared to a prioritization approach.

The target compound usually has no data for the endpoint for which a read-across conclusion is made. Often, it is characterized only by its chemical structure and its physicochemical properties. To date, it is not possible to perform the analogy concept without structural information. For compounds of indeterminate compositions such as multi-constituent substances and substances of unknown or variable composition, the RAAF can in principle also be applied but need additional

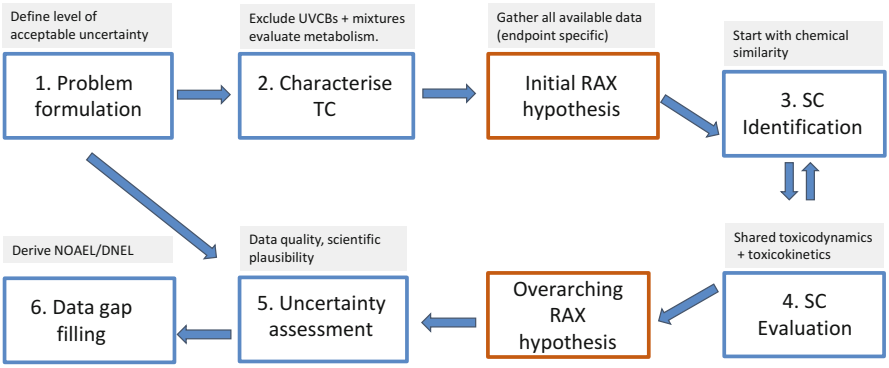

Fig. 2 Schematic overview on the six main assessment steps within a read-across approach (adopted from Escher et al. 2019, with kind permission from Springer Nature). The problem formulation and the characterization of the target compound (TC) lead to an initial read-across hypothesis, which then guides the selection of relevant source compounds (SCs). SCs are often defined based on structural and physicochemical similarity compared to the TC. Step 4 evaluates the toxicokinetic and dynamic properties of the SCs that leads to an overarching read-across hypothesis. The read-across hypothesis, e.g., defines which toxicological effect has to be read across the compounds. In case of property cliffs, the initial RAx hypothesis is modified; some SCs might be excluded to include new SCs. Steps 5 and 6 include the data gap filling process and the assessment of the remaining uncertainty

assessment steps (ECHA 2017). This chapter will illustrate RAx concepts for mono-constituent compounds, with a defined chemical structure. As a **second step** for the read-across assessment, all available data that might be useful to characterize the target compound have to be gathered. Which data to consider is endpoint dependent, i.e., it might be reasonable to include data on chemical and biological reactivity to address genotoxicity/sensitization, e.g., derived from experimental or QSAR models. For other endpoints like reprotoxicity, this information might be considered to be less relevant.

Let's assume that we like to do a read-across to assess toxicity after repeated exposure for the substance diethylamine (CAS 109-89-7). Diethylamine is a basic, water-soluble, colorless liquid with an ammonia-like odor. Its high vapor pressure of 25.9 kPa[1] indicates that humans will be mainly exposed via inhalation. Application of the pure substance results in corrosiveness to the eyes and skin and potentially also to the respiratory tract. A special concern for genotoxic activity is not predicted using the rule-based QSAR models of the OECD QSAR toolbox[2]. The initial read-across hypothesis could therefore be that structurally similar compounds, e.g., other volatile alkylamines, will be good candidates for source compounds. Here the structural similarity but also the physicochemical properties support the read-across hypothesis.

Identify Source Compound(s) Starting with Structure Similarity

Taking this initial hypothesis into account, similar source compounds are identified (**step 3**). Several tools can be applied to search for structurally similar compounds, e.g., noncommercial applications like the OECD QSAR toolbox, AMBIT[3], ToxRead[4], and GenRa (Helman et al. 2019). Typically, the 2-D structure of the TC is entered in a computer-readable format (e.g., as Smiles or INCHI code), and a molecular fingerprint is generated (e.g., Morgan fingerprint; Toxprints, MACCS fingerprint, etc.).

For a structural definition of the source compounds, the molecular fingerprint describes the presence or absence of structural features, as binary string. Several algorithms can be used to calculate a degree of similarity between a pair of molecular fingerprints, like the Tanimoto or Dice coefficients (Bajusz et al. 2015; Murgia and Villasenor 2003). These algorithms have in common that the number of unique molecular features of molecule (a) and (b) is set in relation to the number of shared molecular features in both (c) (Fig. 3). In the present hypothetical example, diethylamine is compared to dimethylamine. The Tanimoto coefficient results in a similarity score of 78% and Sorensen-Dice in a similarity score of 88%. This example illustrates that the similarity score is a relative measure, dependent on the applied fingerprints and algorithm.

A structural similarity search might result in a list comprising many different analogues. In case of diethylamine, high similarity scores are obtained for analogues that differ with regard to the nature of the alkyl substituents, the number of alkyl substituents, or the salt. They might all be good candidate source compounds, or a subset (A to C) might be most appropriate (Fig. 4).

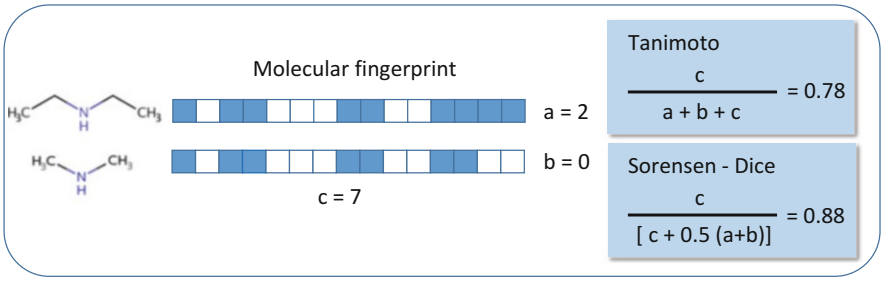

Fig. 3 Calculation of similarity coefficients between dimethyl and diethylamine based on a hypothetical molecular fingerprint (*blue* present, *white* not present); (**a**) number of unique structural features in dimethylamine; (**b**) number of unique structural features in dimethylamine; (**c**) number of shared structural features

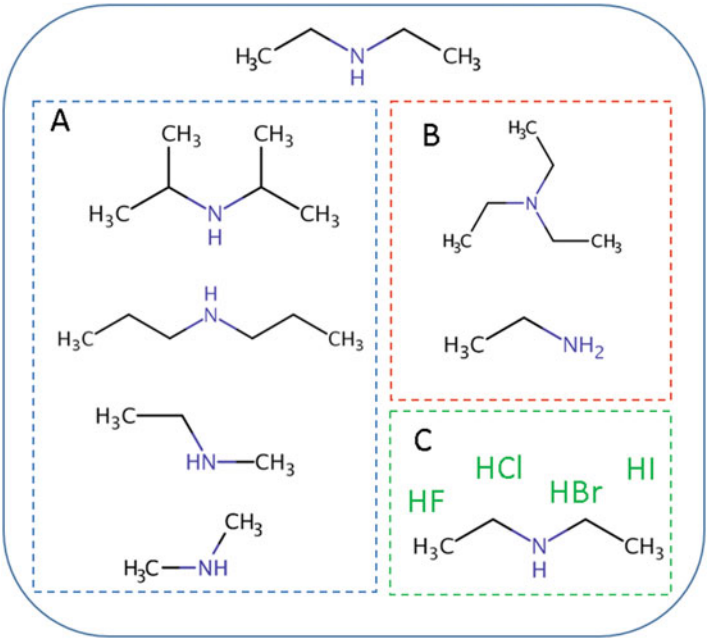

Fig. 4 Initial list of structural analogues for the target compound diethylamine (top structure). (**a**) secondary amines with different alkyl-side chains (C1 to C4); (**b**) different number of substituents ranging from mono- to triethylamines; (**c**) diethylamines with different counter anions

The analogues will have to have high-quality endpoint data for which the read-across is performed. Recently, Pawar et al. reviewed existing databases that are intended to be used in in silico chemical or drug safety assessment (Pawar et al. 2019). Some of the abovementioned tools like the OECD QSAR toolbox or GenRa[5] allow the user to search for toxicological data. Read-across is most often performed for higher tier endpoints like toxicity after repeated exposure. High-quality databases

with subacute, subchronic, and chronic studies are RepDose (Bitsch et al. 2006), ToxRef (Martin et al. 2009), and Hess (Abe et al. 2012), as well as eTox (Sanz et al. 2017) and CPDB (Gold et al. 1999, focus on chronic and cancer studies).

Source Compound Evaluation

The initial list of analogues is the starting point of **step 4** "source compound evaluation." **Step 4** aims to identify from the list of structurally similar substances those source compounds that exhibit similar toxicokinetic and toxicodynamic properties compared to the target compound.

The objective of the toxicokinetic assessment is to identify potential differences in compound bioavailability in the human/animal organism. Since the bioavailable concentrations will interact with tissues/cellular targets and cause systemic toxicity, toxicokinetic studies on absorption and excretion are needed to compare toxicological potencies within the grouped sources and to extrapolate to the target compound. Differences in tissue distribution might help to explain the presence/absence of effects in target organs between grouped compounds. Furthermore, the impact of metabolism needs to be identified. Metabolism plays an important role, as biotransformation might lead to more toxic (e.g., phase 1 metabolism) or less toxic (e.g., conjugation reactions in phase 2) metabolites. In the rare cases where the source compounds cause their effects by the formation of a common "toxic" metabolite, metabolite formation is at the heart of the RAx hypothesis, and its kinetics will be the basis for extrapolation. However, in vivo ADME (absorption, metabolism, distribution, and excretion) data do not belong to the standard regulatory test requirements, e.g., under REACH, and are, therefore, rarely available for chemicals. Surrogate parameters (e.g., water solubility, octanol-water partition coefficient (logPow)) may be used for an initial assessment of potential risk factors, such as bioaccumulation in humans.

The toxicodynamic assessment aims to show that the target compound causes similar toxicological effects as the source compounds. Ideally, source and target compounds share the same mode of action, e.g., as illustrated by an adverse outcome pathway (AOP).

Adverse outcome pathways (AOPs) describe in a linear, transparent, and quality-controlled linkage of key events within a biological process leading to an adverse effect in the organism. After chemical perturbation, the AOP links a molecular initiating event (MIE) to key events on the cellular and organ level, which then progress to the adverse outcome (AO, Leist et al. 2017). Although the concept of AOPs was only introduced in 2010, over 300 AOPs have already been established or are under development (AOP wiki[6]).

A well-known example are acetylcholinesterase (AChE) inhibitors, in particular carbamates and organo(thio)phosphates (OPs), which are characterized by their common $PO_{4-x}S_xR3$ motif. Carbamates are classical pseudo-irreversible AChE inhibitors, which bind to the AChE catalytic site covalently via carbamylating a conserved serine residue. Inhibition of AChE prevents the degradation of the

neurotransmitter acetylcholine, leading to overstimulation. Chronic toxicity resulting from OP exposure ranges from cholinesterase inhibition in plasma, erythrocytes, and brain tissue to the appearance of clinical signs of long-term damage to the central nervous system (CNS) as well as peripheral nervous system (Ecobichon et al. 1990). This is an ideal situation, where the read-across can be based on the MIE (AChE inhibition) and draw a link to the observed adverse in vivo outcome (AO, damage to central nervous system). Often the in vivo studies, like subchronic studies in rodents, only provide the information about the adverse effect. The different molecular interactions (MIE or KEs) leading to the observed effects are often not known. The situation is in addition complicated by the fact that in vivo animal data of the source compounds not always follow the same study design. Data variance may be caused by the use of different species, strains, and exposure regimes, e.g., in terms of dose selection and dose intervals.

The evaluation of source compounds might lead to the conclusion that some structural analogues are dissimilar with regard to their toxicokinetic and dynamic properties. In this case, the initial RAx hypothesis failed. These analogues have to be excluded, and, as an iterative loop, potentially new criteria have to be defined to identify more suitable analogues.

In case that the analogues show similarity, this leads to the overarching RAx hypothesis, based on the observed effect data. Examples are as follows: all source compounds are liver toxicants in vivo that induce cholestasis, or all analogues cause pulmonary irritation progressing to inflammation, etc.

Data Gap Filling and Uncertainty Assessment

The next assessment steps comprise the uncertainty assessment (**step 5**) and the data gap filling (**step 6**). As a read-across is a method of prediction, ideally based on an interpolation, the derived result always inherits an uncertainty, which has to be addressed. One main source of uncertainty is the number of analogues with endpoint data. With a decreasing number of source compounds, the similarity assessment with respect to toxicological properties becomes more and more difficult, in particular within the one-to-one extrapolation (analogue approach). Another aspect that contributes to the uncertainty of the assessment is the quality, relevance, and accuracy of the in vivo endpoint data. Several publications have proposed schemes to address the uncertainty of each step of the read-across assessment, e.g., in a semiquantitative way (Blackburn and Stuard 2014; Schultz et al. 2015; EFSA 2018). In addition to uncertainty analysis of each individual assessment element, finally, the impact of the combined aspects has to be weighted and an overall uncertainty estimate has to be given.

The data gap filling (**step 6**) is done for the endpoint that is read across. In case of qualitative predictions, e.g., is the target genotoxic allows only the conclusion it is or it is not. The weighting of conflicting in vitro and in vivo evidence will require a weight of evidence approach, and approaches already well described for the

assessment of compound-specific experimental data can be applied (Benfenati et al. 2019; EFSA 2017).

In case of a quantitative read-across, e.g., derivation of a no-observed-adverse-effect level (NOAEL), the user has to decide on the most appropriate approach. Three options are available:

(a) A worst-case estimate: The lowest value of all available analogues is chosen and read across to the target compound. This option is often preferred as it follows the precautionary principle of human risk assessment.
(b) A regression analysis: The endpoint values of the grouped analogues follow a consistent trend, and the values of the target compound can be derived from the regression.
(c) Local similarity or nearest neighbor approach: One of the analogues is closest to the target compounds with regard to its toxicological properties, and its values are used for read-across.

Moving Toward Mechanistic Risk Assessment Using In Vitro and In Silico Models

In the previous sections, we have seen that read-across is dependent on the availability of source compounds with relevant in vivo endpoint data. The main challenge within the assessment is to provide convincing evidence on the shared toxicodynamic and kinetic properties within the grouped compounds (Schultz and Cronin 2017). To further enhance the quality of read-across cases and to reduce its uncertainty, it can be useful to support the read-across hypothesis by mechanistic and kinetic evidence from new approach methodologies (NAMs, Zhu et al. 2016). NAMs in this context stand for human in vitro and in silico models. The application of in vitro assays in the context of an integrated approach to testing and assessment (IATA) goes along with new challenges. These challenges include, e.g.:

- Assay-specific aspects like the robustness, reproducibility, and applicability domain of the individual assays (Krebs et al. 2019, 2020)
- The scope of NAM testing needed to address the endpoint under evaluation
- The relevance of different assays/the IATA for the endpoint of concern
- Characterization of the uncertainties associated with the in vitro to the in vivo extrapolation
- Many more

Recently, the EUTOXRISK project developed a concept of an IATA with the aim to integrate NAMs into a RAx assessment for the endpoint repeated dose toxicity (Escher et al. 2019, Fig. 5). As before, the read-across hypothesis is built on the lead effect being observed within the available in vivo data from the source compounds. In this context, lead effect is defined as adverse effects that are observed at the lowest-observed-adverse-effect level (LOAEL). The source compounds are then

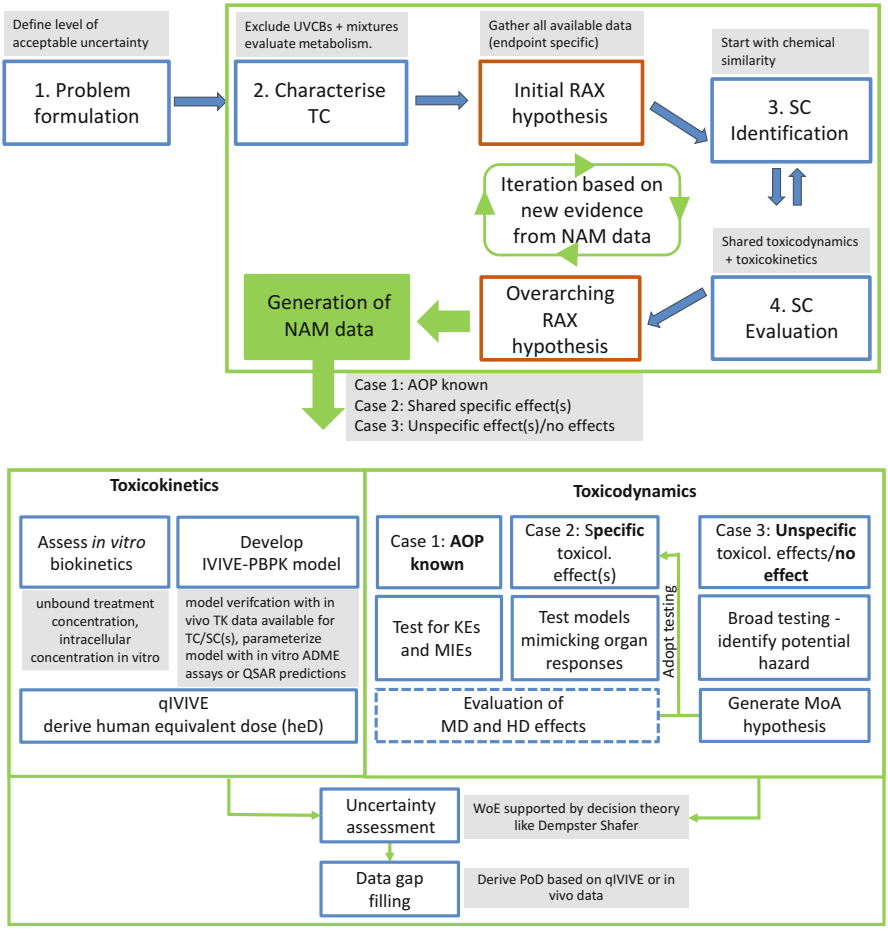

Fig. 5 The EUTOXRISK project scheme on the integration of NAMs into a RAx assessment. Steps in which NAM data can be involved are indicated in green. (Adopted from Escher et al. 2019, with kind permission from Springer Nature). Driven by the overarching read-across hypothesis, NAMs are generated in a systematic way to better characterize the mode of action (MoA, toxicodynamics) and the differences in bioavailability (toxicokinetics) within the grouped compounds. The quantitative in vitro to in vivo extrapolation (qIVIVE) uses an IVIVE-PBPK model and the unbound free intracellular concentration from the in vitro assays to model a human equivalent dose (heD). The uncertainty assessment has to address each individual step of the testing strategy, and decision theories like Dempster-Shafer might be useful to support the integration of data from different in vitro assays. Data gap filling is possible based on the available in vivo endpoint data of the source compounds that are based on the derived heD from the qIVIVE approaches

tested in a systematic way according to this hypothesis to better characterize their hazard with selected in vitro and in silico models. The concept distinguishes three cases.

Case 1: The source compounds share lead effects for which AOPs are known. The well-characterized AOPs are then used to identify relevant in vitro assays to test for the shared mechanisms of action within the grouped compounds and get qualitative as well as quantitative information.

Case 2: The source compounds share specific lead effects, but AOPs are not yet known. Relevant in vitro assays will be selected that cover as far as possible the target organ physiology also with the aim to demonstrate that the grouped compounds share the same mechanism of actions.

Case 3: All the source compounds show low toxic and/or not specific toxicological. In this case, it is recommended to use a broader in vitro testing battery to detect potential dissimilarities between the grouped compounds that may alert for a specific toxicological concern for the target compound.

The screening of the hazard of the grouped compounds with NAMs reduces time and costs compared to in vivo testing, so that more analogues can be tested and the impact of minor structural changes can be investigated. The selection of source compounds can thus be expanded to analogues without in vivo endpoint data.

As stated above, most chemicals don't have in vivo ADME data (Wetmore et al. 2015). NAM-based approaches allow to address the toxikokinetic properties within the grouped compounds by using in vitro to in vivo extrapolation linked to physiologically based kinetic (IVIVE-PBPK) modeling (Arora et al. 2020). PBPK models use physiological (systems) data to estimate the distribution of the compounds within the human/animal organism and indicate the resulting bioavailable concentration, e.g., in certain target organs or plasma. IVIVE is used as indication that in silico and in vitro parameters are used to parameterize these PBPK models, namely, binding to plasma proteins, the blood/plasma ratio, and the intrinsic clearance in primary human hepatocytes. IVIVE-PBPK models are open source models like the httk model developed by US EPA (Pearce et al. 2017a, b), TK-plate developed by EFSA (Wiecek et al. 2019), or commercial models like the SIMCYP™ PBPK Simulator (Certara[7]).

The evidence from the NAM data is used to establish a strong link to the overarching RAx hypothesis. Ideally, concordance between the assays is observed allowing to conclude on a shared mode of action (MoA). For some endpoints, several KEs and MIEs might have been tested, so that data integration and interpretation might get difficult. Decision tools like the Dempster-Shafer theory (DST) are suggested for data integration. The DST is a rigorous decision-theory approach that can be used to combine multiple sources of evidence to obtain a weight-of-evidence prediction by quantitatively accounting for the reliability of each of the sources being combined. In addition, the DST provides a quantitative estimate of the associated uncertainty.

Although the IATAs and NAM models work with a lot of assumptions, it has been shown in first case studies that they are useful to estimate (dis)similarities and trends within a read-across assessment (OECD 2020a). The experiences of these case studies also contributed to a reporting template (OECD 2020b).

In conclusion, RAx is a very useful method/tool to close data gaps in human risk assessment (also for regulatory purposes), and integration of NAM data has shown to enhance the quality of the assessment by providing data on shared mechanistic and kinetic properties.

Cross-References

- ▶ Assessment of Mixtures: Bridging Principles and Other Approaches
- ▶ Computer-Based Prediction Models in Regulatory Toxicology
- ▶ Default Factors in Quantitative Risk Assessment
- ▶ Integration of Advanced Technologies into Regulatory Toxicology
- ▶ Psychoactive Designer Drugs: Classes, Mechanisms, and Regulation
- ▶ Toxicokinetic Models
- ▶ Uncertainty Analysis in Exposure Assessment-Relevance for Toxicological Risk Assessment

Notes

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Green and Sustainable Chemistry as Regulatory Levers

36

Michael Schwenk

Contents

Abstract

The increasing environmental pollution, industrial accidents and chemical-induced exposures, and resulting disease led to the concept of a green chemistry in 1998. It proposes to design chemicals in a way that no hazardous reagents, products, or exposures would occur. The concept is of outstanding importance,

M. Schwenk (✉)
Tübingen, Germany

Formerly: Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

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when considering, that all aspects of human civilization including clean water, global nutrition, agriculture, housing, household goods, and medication all depend on functioning chemical designs, industrial production, and on recycling/regeneration efforts. The UN-Sustainable Development Goals (SDG), adopted in 2015, will have to rely on green and sustainable chemistry tools. International and national organizations adopt green and sustainable chemistry into their regulations.

Keywords

Sustainability · Green chemistry · Pollution · Toxicology · Chemical safety · Agenda 30 · Development · Chemical synthesis

Introduction

Regulations for the protection of health and to reduce specific risks have been established since long. But when new types of environmental health problems were noticed, such as the London smog episode of 1952 (Bell et al. 2004), techniques to analyze the causes in detail or to avoid hazardous exposures where not yet available.

The widespread pollution of outdoor air, aquatic systems, and soils (brownfields) became evident in the 1960s, and with it the causal relationship between chemical exposures and occupational/environmental disease was investigated in more detail. State agencies, industry, and the public were in alert. Main stream politics showed little interest initially, being preoccupied with other problems such as the arms race. Nevertheless, environmental protection became a topic and concerned people joined green movements with the aim to prevent pollution and reduce environmental disease. At the same time, technical solutions to limit emission became available or affordable.

Before the 1970s, chemistry students were proud to work in a laboratory that smelled of solvents. But that changed. The green chemistry concept of the 1990s (Anastas and Warner 1998) was a valuable contribution to find ways for future-oriented chemical design and sustainable products. In 2015, the UN-Agenda 30 with its 17 sustainable development goals (UN 2015) provided an internationally accepted roadmap. Basically, green chemistry and sustainable chemistry have the same goal and their concepts support sound regulations for the protection of health and of the environment (Kummerer 2017; Anastas and Zimmerman 2018; OECD 2020, Umweltbundesamt 2020).

The present contribution describes the cumulating problems, and the developments, that led to the green chemistry concept, depicts areas where green chemistry can support a sustainable development, and suggests the idea of integrating toxicology into this concept.

Developments Before the Area of Green Chemistry

Early Industrial Chemistry

Since the beginnings of industrial chemistry, more than 200 years ago, synthesis processes were stepwise improved to increase output, product variety, and profit. Rare and valuable chemicals were recovered, when possible. Rules were developed to fight accidents in chemical plants. But other areas were initially neglected, such as workers health, waste production, emissions, and environmental pollution.

Pollution and Accidents (1965–1990)

The period from about 1965 to 1990 was characterized by major chemical accidents and increasing awareness of pollution and the need for more chemical safety.

Pollution of Environmental Media

Outdoor Air
Emissions from incineration processes were found to cause health risks through fine particles, acids, and many other combustion- and photochemical reaction products further polluting the clean air.

Ozone Hole
Chlorofluorocarbons were found to deplete stratospheric ozone and its filter function against harmful solar ultraviolet light.

Surface Water
Domestic and industrial wastewater often went unfiltered into rivers, carrying pollutants into river beds, lakes, and oceans.

Ground Water
Chemicals from landfill waste and agricultural applications were found to leak into the ground and to contaminate ground water, the important source for drinking water.

Dumping Sites and Soil
Some areas, formerly used as dumping sites for toxic substances were converted to residential areas (e.g., Love Canel in the USA). Moreover, benzo(a)pyrene and toxic metals, emitted during combustion processes into ambient air, were found to contaminate the soil of (former) industrial neighborhoods.

Indoor Air

Urea formaldehyde foams, widely introduced to improve the thermal insulation of homes, were found to release formaldehyde, causing intolerable indoor concentrations. Solvents of paints and adhesives as well as volatile wood preservatives (e.g., pentachlorophenol), sealing joint material (polychlorinated biphenyls), insecticides (e.g., DDT), and asbestos caused partly high indoor air concentrations.

Consumer Goods

Just to give one example: Carcinogenic nitrosamines were found as side products in rubber-based soothers.

Pollutants of Major Concern (1965–1990)

Persistent Organic Pollutants (POP)

A whole range of cyclic and aromatic chlorinated hydrocarbons were used as pesticides (e.g., DDT) or technical applications (e.g., PCBs). Even more, the polychlorinated dibenzodioxins (PCDDs) which had no technical application were identified as side products of some synthesis processes and of waste-incineration reactions. Such substances are today labeled as POP, because of their very long persistence, widespread distribution around the globe, and accumulation in the food-chain.

Toxic Metals

In the 1950s, toxic metals had still applications as pharmaceuticals, paints, plumbing, cosmetics, and antimicrobial agents. For example, tetraethyl lead was the common antiknock additive of gasoline until the 1980s. Phosphate fertilizers and sewage sludge were found to be contaminated with cadmium, a toxic metal that is taken up by agricultural plants. Mercury from industrial processes entered a bay in Minamata and the mercury was converted by microorganisms to neurotoxic methylmercury that gradually accumulated in fish. The substance poisoned the fish-eating population. Reduction of global mercury usage was later regulated in the Minamata Convention.

Polycyclic Aromatic Hydrocarbons

Benzo[a]pyrene is the lead toxic substance of polycyclic aromatic hydrocarbons. It is formed during coal combustion processes, and found as contaminant that persists very long in soil around industrial areas, fire places, and brownfields.

Endocrine Disruptors

A number of environmental chemicals (e.g., phthalates, polychlorinated biphenyls) used, e.g., as additives to plastic materials were found to exhibit hormonal effects. They may disrupt reproductive physiology of aquatic organism. The role for humans is not entirely clear.

Chlorinated Volatile Solvents

This group of solvents has the practical advantage of evaporating very quickly. They were used in paints, glue, and cleaning liquids by occupational users and non-professionals, often indoor. They were found to induce a range of substance-specific chronic-toxic disorders. Being volatile, they also disturb atmospheric chemistry.

Industrial Accidents (Examples)

Seveso

Due to accidental overheating of a chemical reaction in a pentachlorophenol-producing plant in northern Italy in 1976 the minor side-product 2,3,7,8-tetrachlorodibenzodioxine was generated at high levels. It was released into the environment, causing widespread pollution.

Bhopal

An accident in a pesticide-producing unit in Bhopal (India) in 1984 released large amounts of a mixture of irritating and toxic agents that caused a disaster with thousands of fatalities in the nearby living population.

Chernobyl

A nuclear power plant went out of control in the Ukraine in 1986 and emitted radioactive products. Beyond the tragedy of the massive regional radioactive contamination, some radioisotopes such iodine-131 and cesium-137 were carried with the atmospheric stream thousands of kilometers. They washed out with the rain and caused long-lasting soil-contaminations in distant regions of Middle-Europe.

Chemical-Induced Disease

In conclusion, chemical exposures were identified to constitute a health risk for workers in the chemical industry, for professional users, and also in the general population. Some of these associations became visible between the 1950s and evident with the publication by the epidemiologists Doll and Peto (Doll and Peto 1981; see also Blot and Tarone 2015). Each toxic chemical tends to have a specific fingerprint with regard to its adverse chronic effects, as exemplified in Fig. 1.

Drug Scandals

Two major incidents with pharmaceuticals concerned exposed pregnant women: Thalidomide, sold in the late 1950s as sedative and anti-nausea medication, caused limb abnormalities in the developing child. The synthetic estrogen diethylstilbestrol-

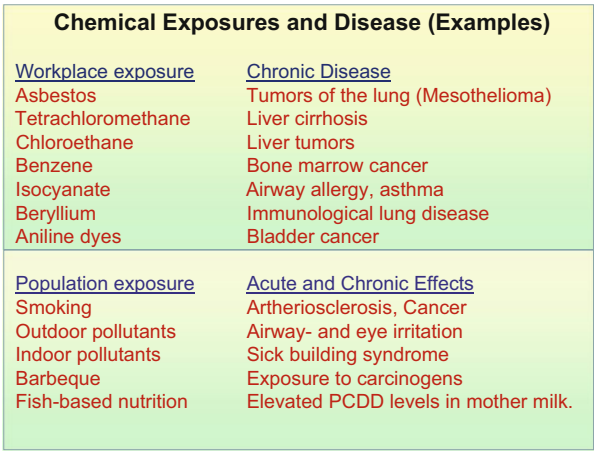

Fig. 1 Examples for causal relationships between occupational exposures or population exposures, and disease

induced vaginal tumors in daughters. This contributed to profound reconsideration of drugs- and chemicals safety in general.

Advances in Chemical Safety

Improved quantification techniques for environmental chemicals and the advances in toxicology made it possible to identify pollution problems, and whistleblowers helped to make these problems known; this later resulted in the "Green Chemistry" concept.

Analytical Chemistry and Clinical Chemistry

Steadily improving analytical separation procedures (often GC, HPLC) together with the advances in instrumental detection methods, like atom absorption technology to detect toxic metals, made it possible to reliably detect and quantify increasingly more anthropogenic chemicals in environmental media and body fluids. The availability of enzyme-linked immunoassays further expanded the detection possibilities.

Toxicological Advances

Due to progresses in biochemistry, analytical chemistry, and molecular cell biology, it was possible to identify mechanisms of acute toxic cell damage and tissue damage as well as chronic organ disorders. The role of reactive intermediates, that are formed during enzymatic biotransformation of some substrates, was recognized and became the key for an understanding of carcinogenic mechanisms of substances like aflatoxin, benzo[a]pyrene, or nitrosamines. Novel in vitro test systems allowed to better study such mechanisms. The roles of gene-regulation, death receptors, and apoptosis

on the one side and of cellular DNA-repair and tissue regeneration on the other side are still today in the focus of research.

Whistle Blowers

Whistle blowers are often annoying and troublesome, and sometimes they are wrong. But whistle blowers were extremely important to promote discussions that resulted in more awareness of the long-time risks of chemicals in the environment and risks associated with exploitation of the global resources. Here some examples:

Rachel Carson, a marine biologist, described in her book "Silent Spring" (Carson 1962), the phenomenon of bioaccumulation of persistent environmental pollutants (e.g., DDT) in the food chain and its negative consequences for wildlife.

Greenpeace: Canadian activists went on a boat trip in the Pacific to demonstrate against nuclear testing in 1971, when the nuclear arms race was in full swing. This peaceful action initiated the Greenpeace organization.

Club of Rome: This highly respected institution published the book "Limits to Growth" in 1972. Based on mathematic models, it convincingly argued that further economic growth and population expansion will deplete the resources on earth.

Ashford and Miller: The book "Chemical exposures, low levels and high stakes" (Ashford and Miller 1991) collected evidence for chemical-associated sensitivities in the population that had been previously described by Cullen as "Multiple Chemical Sensitivity" (Cullen 1987).

Information Systems

Until the early 1970s, scientist informed themselves from books, conferences, and scientific journals. This changed. The World Health Organization started editing information on toxic substances in 1976. The Hazardous Substances Databank (HSDB) of the US-National Library of Medicine became available for users in 1978, and Medline started to offer access to its databank in the 1980s. Many other high-quality toxicology databanks followed and started to get accessible via the Internet. The fast access to original publications and to condensed secondary information about chemicals and their hazards provided an invaluable basis for evidence-based regulations.

Harmonization of Regulations

As a consequence of the awareness of the pollution problem scientific committees, national agencies, and international organizations were founded. Prevention started to become important. Major pollution problems, as described above, were increasingly regulated by banning, restricting, or limiting the use and/or release of hazardous substances.

Initially, it took a long time until harmonization of test systems and respective test guidelines were developed. With the years, the guidelines of OECD for chemicals and ICH for therapeutic drugs have developed to be backbones for hazard and risk assessment.

Green- and Sustainable Chemistry

Green Movements

Green movements initially emerged from protests against environmental pollution. "Greenpeace"may have played a pioneer role. Regional green movements started in the mid-1970s; some converted to political parties, others are active as NGOs.

"Green Chemistry" Beginning and Mission, Expansion

Outsiders often assume, that "green chemistry" simply refers to the use of biomass as energy source and organic raw material or refers to the application of bioengineering methodology; others may assume that it refers to "Organic Movement," the proponents of which tend to avoid synthetic chemicals. But green chemistry is much more.

In "The Slow Birth of Green Chemistry" (Amato 1993), the author described the ongoing pollution and the necessity for an environmentally responsible chemistry. Not much later (1996) came the EPA Green Chemistry Award that has successfully continued until today (EPA 2020a). The early cooperations were described by Goehl in 1997 (Goehl 1997). Then appeared the book "Principles of Green Chemistry" by Anastas and Warner in 1998 (Anastas and Warner 1998). The authors defined 12 principles of green chemistry and provided discussions and outlooks for each of them. Table 1 lists the 12 principles in their original form *(*Anastas and Warner 1998*)* and in a nice short version that is found on a green chemistry website of the American Chemical Society (ACS 2020a).

In essence, the 12 principles express, that chemical synthesis processes should be designed in a way, that avoids waste, minimizes energy input and avoids products that are toxic to humans or the environment.

Anastas and his group continue to intensely promote the green chemistry concept. The 12 principles developed to a paradigm of green chemistry (Anastas 2018). Thus, the "green" idea transformed into a constructive practical guidance for chemistry. The US-EPA has been involved from the beginning and still plays a lead role (EPA 2020b). The American Chemical Society (ACS) promotes the concept among scientists in academia, industry, and regulation (ACS 2020b). Large companies of the chemical industry tend to cooperate as a contribution for the future.

Sustainability Movements

Sustainability can be considered as a conservative concept that has routes in forestry. Tree saplings were planted to regenerate deforested areas, with the idea to provide next generations with grown trees. Sustainability has long been neglected in the twentieth century. So it was a milestone, when the United Nations (UNCED) "Rio Declaration on Environment and Development" (1992) used "sustainability" as its key plan to protect

Table 1 The left columns shows the 12 principles of green chemistry, as published by Anastas and Warner in the booklet "Green chemistry- theory and practice" (Anastas and Warner 1998). The right panel shows a short version, according to the American Chemical Society (ACS 2020b)

No	Principles, Original Version (Anastas and Warner 1998)	Short Version (ACS 2020b)
1	It is better to prevent waste than to treat or clean up waste after it has been created	Waste Prevention
2	Synthetic methods should be designed to maximize incorporation of all materials used in the process into the final product	Atom Economy
3	Wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and the environment	Less Hazardous Synthesis
4	Chemical products should be designed to preserve efficacy of function while reducing toxicity	Design Benign Chemicals
5	The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and, innocuous when used	Benign Solvents & Auxillaries
6	Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure	Design for Energy Efficiency
7	A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable	Use of Renewable Feedstock
8	Unnecessary derivatization (use of blocking groups, protection/deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste	Reduce Derivatives
9	Catalytic reagents (as selective as possible) are superior to stoichiometric reagents	Catalysis (vs. Stoichiometric)
10	Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment	Design for Degradation
11	Analytical methodologies need to be further developed to allow for real-time, in-process monitoring, and control prior to the formation of hazardous substances	Real Time Analysis for Pollution Prevention
12	Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires	Inherently Benign Chemistry for Accident Prevention

the earth and its resources. It can be mentioned, that the Rio declaration had given the mandate for safer use of toxic chemicals, thus establishing the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) in the Agenda 21, para. 19.27: "*A globally harmonized hazard classification and compatible labelling system, including material safety data sheets and easily understandable symbols, should be available, if feasible, by the year 2000*" (UN 1992).

The United Nations Sustainable Development Agenda 30

In the year 2015, the 17 United Nations Sustainable Development Goals (SDGs) were launched as core part of the Agenda for Sustainable Development 2030, the Agenda 30 (UN 2015). The SDGs are based on the Agenda 21 of the Conference on Environment and Development (Rio de Janeiro, 1992). They address the most urgent topics for a global sustainable development (Fig. 2, left panel). They constitute the consensual results of intensive consultations of stakeholders from governments, NGOs, and other groups. The UN expects that the goals will stimulate actions that will be of critical importance for the future of humanity and the planet (UN 2015).

"Sustainable Chemistry"

The 17 UN goals for sustainable development have a wide coverage. The term "sustainable chemistry," stands for more than the term "green chemistry" that has its focus on chemical production/synthesis processes. "Sustainable chemistry" includes synthesis, but has a second focus on processes, not directly related to synthesis and including innovations that allow to reach the UN sustainability goals.

The term "sustainable chemistry" may have the additional advantage of not being associated with a political movement/party ("the greens"), so may receive a wider acceptance. But in principle, both have the aim of a "chemistry for a sustainable world."

UN Sustainable Development Goals (SDG) and Chemistry

UN-Goals (2015)	Sustainable Chemistry Approaches (examples)
1: No Poverty	1. Local solar panels, sanitation products.
2: Zero Hunger	2. Water desalination, adapted agrochemicals.
3: Good Health and Well-being	3. Drug design, healthcare products.
4: Quality Education	4. Courses in environmental sciences.
5: Gender Equality	5. Equal gender chances in the profession.
6: Clean Water and Sanitation	6. Water regeneration and purification items.
7: Affordable and Clean Energy	7. Improved energy yield and energy storage.
8: Decent Work and Economic Growth	8. Chemical design for a circular economy.
9: Industry, Innovation and Infrastructure	9. Novel materials for each purpose.
10: Reduced Inequalities	10.International science networks and exchange.
11: Sustainable Cities and Communities	11.Community hygiene products, clean water.
12: Responsible Consumption and Production	12.Common sense for stable flow equilibria.
13: Climate Action	13.Innovative energy systems.
14: Life Below Water	14.Respect for nature and its preservation.
15: Life on Land	15.Sustainable use of raw materials.
16: Peace, Justice and Strong Institutions	16.Prohibition of chemical weapons.
17: Partnerships for the Goals	17.Cooperation with UN, OECD etc.

Fig. 2 Chemistry and the Sustainable Development Goals of the United Nations. The figure lists on the left panel the 17 UN global sustainable development goals (SDG) of the Agenda 30 (UN 2015), and on the right panel some examples for possible/necessary contributions of chemistry to achieve the goals. After IUPAC (2017), modified

It is evident that chemistry must play a key role in actions for the future: To minimize pollution and harmful exposures by chemical intermediates and products, as well as providing tools to achieve the goals of clean energy, clean air, clean water, and a clean environment. Chemistry will be of special importance for research, design, production, and the safe disposal of existing and novel chemicals. Figure 2 (right panel) depicts some of the fields, in which chemistry will be needed to actively support the SDGs.

Basically chemists may appear not to have specific responsibility for the sustainable development of the world. But they do. Only chemists have the knowledge about synthesis processes and the properties of their products, on which the non-experts will rely.

The huge complexity of the human activities affecting atmospheric chemistry, water quality, ocean life, climate, soil pollution, and depletion of essential natural resources makes very clear that sustainable chemistry is a global task.

Many regulating agencies and scientific organizations soon adopted the "Green chemistry" concepts in their programs in 1995–2000. The International Union of Pure and Applied Chemistry (IUPAC) that represents the chemical societies of many countries, developed a working group on green chemistry which culminated in a Special Issue of the Journal Pure and Applied Chemistry in the year 2000 with an introductory contribution by Tundo et al. (Tundo et al. 2000). IUPAC's Interdivisional Committee on Green and Sustainable Chemistry is very active to promote green chemistry concepts worldwide (IUPAC 2020).

The Organization "Strategic Approach to International Chemicals Management" (SAICM), adopted in 2006 is a policy framework to promote chemical safety around the world.

Chemical industry provides signals that it goes in line with SDG (Axon and James 2018).

OECD, that is the lead organization for toxicological guidelines, also discusses and promotes green and sustainable chemistry (OECD 2020).

The UN-Organization for the Prohibition of Chemical Weapons (OPCW) is assigned to achieve chemical disarmament, including destruction of stored chemical weapons and the banning of production or use of chemical weapons. It also has to control potential precursors of known chemical weapons (a sustainability goal in itself), and supports cooperation in sustainable chemistry to strengthen peace and security (OPCW 2018).

Regulatory Systems like the European REACH have goals in sustainable chemistry (Umweltbundesamt 2020). Many national agencies with responsibility for chemical safety have programs to support green and sustainable chemistry.

Green Chemistry Methods

"*Metrics*" provides an instrument to analyze with suitable parameters all steps of the production process with regard to energy-use, atom economy, and adversity of reactants and products as an objective bases with the aim to further optimize chemical reaction processes (Tabone et al. 2010; Sheldon 2018). Green chemistry metrics have been used to improve the efficiency in reaction design (Sheldon 2012).

Chemistry's traditional commitment to "quality" also promotes improvements, as described by Murphy for the pharmaceutical drug ibuprofen (Murphy 2018). Ideally, metrics would include toxicity/tolerability data, an analysis of the life-cycle, benefit/cost of recycling and environmental cost of dumping in landfills.

Catalysts have a central role in green synthesis. The potential for optimization can be seen, by comparing the historic total chemical synthesis of cholesterol in 1951/52 by Woodward (Woodward et al. 1952) with the biochemical pathway in cells. The Woodward synthesis involved 40 reaction steps, including laborious stereoselective reactions, harsh conditions such as heat, acids, and various metal catalysts. Animal cells assemble the cholesterol molecule from acetyl-CoA units in about 40 enzymatic steps at 37 degrees Celsius and neutral pH, using enzymes as catalysts and coenzymes/cofactors as supporting agents in a compartmented, regulated cellular environment. It can be expected that biocatalysts, biosimilar synthetic catalysts and biomimetic techniques may become more important for chemical synthesis in the future (Sheldon and Woodley 2018).

Education is of central importance, in order to train students in systems thinking (Mahaffy et al. 2019) and to support creation of truly sustainable technologies (Cannon and Warner 2011). Courses on green chemistry are regularly offered on national and international levels. IUPAC has taken a lead role in organizing courses in different continents thus generating an incentive for scientific and technical advances. Prices for green science and for green industrial performance are awarded by various institutions and organizations, such as the US-EPA (EPA 2020b) and IUPAC.

The Complexity of Sustainable Decisions

The global problems of toxic exposures and pollution have recently shifted from chemical-industry production sites and products, to new types of problems such as side effects of energy production (CO2, depletion of fossil reserves), animal farming (depletion of soil nutrients, pesticides and resistance to pesticides, release of methane into the atmosphere), combined with poor waste management, insufficient waste chemistry, and continuing exploitation of natural resources.

New types of pollution become visible, such as microplastic in the environment and aquatic media, and airborne engineered nanoparticulate materials of different composition (Gubala et al. 2018). The exploitation of novel types of raw materials (manganese nodules, methane clathrate) will be associated with new risks for aquatic systems and climate.

The sustainability goals of the Agenda 30 are interconnected. Decisions that support one goal may be of disadvantage for other goals. Thus, the idea of using renewable feedstock and raw material has been widely considered as a breakthrough of the sustainability-goal that would help to reduce worldwide carbon dioxide emissions. Subsidies are provided to use forests and grown soy as raw material sources. But this aggravates the worldwide deforestation, the loss of farmland, and the depletion of sweet water reserves.

Similarly, increasing the lifetime of chemical products may appear to be a requirement for sustainable chemistry. However some long-living chemicals and composite materials have the disadvantage of being difficult to recycle and/or being resistant to decomposition when carried into the environment, where they may cause long-lasting pollution of unknown consequences.

Therefore, integration of system-thinking with anticipation of the long-term consequences will be increasingly important in general, and also for a green and sustainable chemistry.

Toxicology for Sustainable Development

Fruitful interdisciplinary cooperation will be required to reach the goals and visions of the UN-Agenda 30. Teaching green chemistry encourages chemists in systems-thinking and in understanding toxicology; this will facilitate communication between chemists and toxicologists (Zimmerman et al. 2014).

A green toxicology should include reduced animal testing, reduced testing time, and saving of resources and of test materials. This goes hand in hand with the trend to replace traditional tests by quality-validated in vitro techniques, such as proteomics, metabolomics, genomics, cell cultures, organoids, and target-oriented high throughput test systems like ToxCast (Dix et al. 2007; Watt and Judson 2018). QSAR and bioinformatics will continue to play a great role. One day it may become possible to define the quantum mechanics features of the ligand-binding pocket of macromolecular receptors and predict its binding characteristics for small molecules, thus providing toxicology-relevant information in the absence of any biological test. However, one can anticipate that a lot of questions will remain unsolved by any in vitro or in silico test and can only be answered by studies in the intact organism, such as the calibration of in vitro assays, aspects of chronic organ toxicity, two generation assays, or detailed toxicokinetic analysis.

The impacts of green chemistry on a green toxicology have been discussed recently (Hartung 2009; Crawford et al. 2017; Rusyn and Greene 2018). It is evident, that the combined expertise of chemists and toxicologist will be required in all aspects of green and sustainable development, whether it concerns innovative chemical products and applications or the reduction of existing and upcoming global pollution problems in a sustainable manner.

Cross-References

- ▶ Aims and Mission of Regulatory Toxicology
- ▶ Computer-Based Prediction Models in Regulatory Toxicology
- ▶ Examination of Acute and Repeated-Dose Toxicity
- ▶ Omics in Toxicology
- ▶ Read-Across Methodology in Toxicological Risk Assessment
- ▶ Regulation and Mitigation of Greenhouse Gases

► Specific Toxicity Tests for Neurotoxicity, Immunotoxicity, Allergy, Irritation, Reprotoxicity, and Carcinogenicity
► Teaching Chemical Safety

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Do Carcinogens Have a Threshold Dose? The Pros and Cons

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Bernd Kaina, Adam D. Thomas, Hartwig Visser, Jan G. Hengstler, and Roland Frötschl

Contents

Abstract

With sound understanding of biological concepts, the notion of threshold effect levels has grown in acceptance especially for electrophile-induced mutations. However, mutagenesis is only one part of the exposure-to-tumor process in chemical carcinogenesis. Another important part is carcinogen-induced cell death and senescence that eliminates or immobilizes heavily damaged and

B. Kaina (✉)
Department of Toxicology, University Medical Center, Mainz, Germany
e-mail: kaina@uni-mainz.de

A. D. Thomas · H. Visser
Centre for the Research in Biosciences (CRIB), UWE Bristol, Bristol, UK
e-mail: Adam7.thomas@uwe.ac.uk; Adam7.thomas@uwe.ac.uk

J. G. Hengstler
Leibniz Research Centre for Working Environment and Human Factors at the Technical University of Dortmund (IfADo), Dortmund, Germany
e-mail: hengstler@ifado.de

R. Frötschl
Genetic and Reproductive Toxicology, Federal Institute for Drugs and Medical Devices, Bonn, Germany
e-mail: roland.froetschl@bfarm.de

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mutated cells, respectively. In the following chapter, we postulate diverse protective mechanisms that may contribute to no-effect thresholds in chemical carcinogenesis. Key mechanisms contributing to threshold doses are carcinogen detoxification, including reactive oxygen species (ROS) scavenging and error-free deoxyribonucleic acid (DNA) repair. Elimination of cells harboring premutagenic DNA lesions by apoptosis and other cell death pathways, reduced proliferation rates within tissues, and the induction of senescence may minimize mutation rates and, therefore, also contribute to threshold dose effects for carcinogenesis.

Keywords

DNA damage · DNA repair · Threshold · Genotoxicity · Mutations · Carcinogens · Alkylating agents · Apoptosis · Cell death · Senescence

Introduction

One of the most significant questions in the field of regulatory toxicology pertains to the concept of threshold dose: do genotoxic carcinogens have a no-effect threshold? The practical ramifications of this question are immense. If there is no evidence of a threshold dose, then limiting exposure to any dose of such chemicals is essential. Conversely, if effect pertains to be threshold, then exposure limitation far below such a threshold becomes an unnecessary burden. The current paradigm assumes that genotoxic carcinogens do not have a threshold dose, whereas tumor promoters and non-genotoxic carcinogens do. Recent evidence for direct genotoxicants has challenged this assumption. There is considerable need for sound understanding of cellular defense mechanisms to substantiate the no-effect levels observed for potent genotoxicants, which is necessary for their acceptance of nonlinearity in risk assessment. In order for accurate conclusions as to the biological relevance of exposure, one has to consider the fate of treated cells. With recent work, we are uncovering the multifaceted cellular responses to genotoxicants, which depend on dose and time and determine cellular fate, i.e., if a cell survives and retains proliferative capacity with or without mutations; dies via apoptosis, necroptosis, or mitotic catastrophe; or remains viable but enters a senescent state. The fate of cells following treatment is not as simple a relationship as the mutation/repair paradigm as a biological basis for thresholds. However, considerations must be made, and our understanding of the signaling mechanisms following treatment must improve because cellular fate, following genotoxic treatment, will have significant bearing on cancer risk. Therefore, the impact of determining cell fate and the signaling mechanisms involved on cancer risk assessment are challenging tasks. Whether different cell fates are elicited at different doses is to be determined. We also have to consider the possibility that at each dose, there will be differing proportions of cells experiencing all possible cell fates, but how this situation contributes to thresholds in carcinogenesis remains to be established.

The question of whether absolute thresholds exist for genotoxic carcinogens cannot be experimentally determined because potential low-dose effects are masked by inherent biological variation. Additionally, due to the complexity of the multi-target model of carcinogenesis, the theoretical deduction of a no-effect threshold is difficult. Thus, *practical* thresholds are *inferred* through experimentally determined dose-response relationships for each endpoint of the carcinogenic process. Therefore, the terms no-*observed* effect level (NOEL), no-observed *adverse* effect level (NOAEL), and no-observed *genotoxic* effect level (NOGEL) are used. With data sets of sufficient statistical power, the parameters point of departure (PoD) and benchmark dose (BMD) are calculated in this respect. Increasing evidence reports nonlinear and even exponential curves for long-term carcinogenicity bioassays in rodents (Waddel et al. 2006). Increased cell proliferation through regenerative hyperplasia at higher doses may potentiate the mutagenicity and carcinogenicity of adducts that remain "dormant" at lower doses, where cell proliferation is comparatively slow (Schulte-Herman et al. 1980). Needless to say, the question of a *true* null effect at low dose will require extensive work.

Tumor Promoters

It is generally assumed that the non-genotoxic mode of carcinogenicity of tumor promoters comes from their ability to modulate signaling pathways, which can lead to stimulation of cellular proliferation or inhibition of apoptotic cell death (Blumberg and Boutwell 1980). Incidentally, there are suggestions that proliferation is stimulated by tumor promoters specifically in cells that were initiated by a genotoxic carcinogen. An example is provided by c-Ha-ras-mutated skin keratinocytes in the two-stage mouse skin cancer model (Parkinson 1985). It is conceivable that genotoxic effects arise indirectly from the promoters' mode of action. For example, the tumor promoter phorbol-12-myristate-13-acetate (TPA) is non-DNA reactive. The promoting effect of TPA is the consequence of interaction with protein kinase C (PKC) and the resulting alteration of signaling pathways under its control. However, TPA indirectly induces DNA damage, sister-chromatid exchanges, and chromosomal aberrations (Dzarlieva and Fusenig 1982) by releasing intracellular DNA-reactive oxygen species (ROS). This DNA damage mode of action (promotion I) may have a mutagenesis threshold, dependent upon the level of ROS production and the concentration of intracellular radical scavengers (Seager et al. 2012). Additionally, the observations of Lutz et al. (1996) indicate that tumor promoters may well be characterized by a threshold dose. Thresholds are dependent upon the mode of action, and whether this threshold is due primarily to protection against ROS-induced damage or through lack of mitogenesis at low doses is unclear. Other tumor promoters stimulate cell division through binding to and activating cell surface receptors. According to the pharmacological receptor concepts, a specific amount of ligand and activated receptor is required to activate a signal pathway to elicit a biological effect. For example, a certain amount of epithelial growth factor

(EGF) is required to activate sufficient receptors to have an impact on cell proliferation and promotion of initiated cells. Therefore, to postulate in this process the existence of a NOEL seems reasonable.

Factors Modifying Carcinogenesis

The hypothesized cytoprotective mechanisms that can theoretically result in a no-effect threshold are summarized in Fig. 1.

The following passages report on the arguments concerning the involvement of each mechanism in no-effect thresholds through the carcinogenic process.

Carcinogen Activation. An important part of the genotoxic effect of many carcinogens is their metabolic activation through enzymes such as cytochrome P450 monooxygenases. The importance of activating enzymes, for provoking a carcinogenic threshold, is observed in the cynomolgus ape. Due to a deficiency in cytochrome P4501A2, cynomolgus apes are resistant to the carcinogenic effects of particular heterocyclic aromatic amines. However, assuming first-order kinetics, it seems likely that even at the lowest dose, each molecule of (pro-) carcinogen could be activated into a carcinogenic metabolite. Saturation of such pathways has only been observed at high dose, thus rendering a low-dose threshold unlikely.

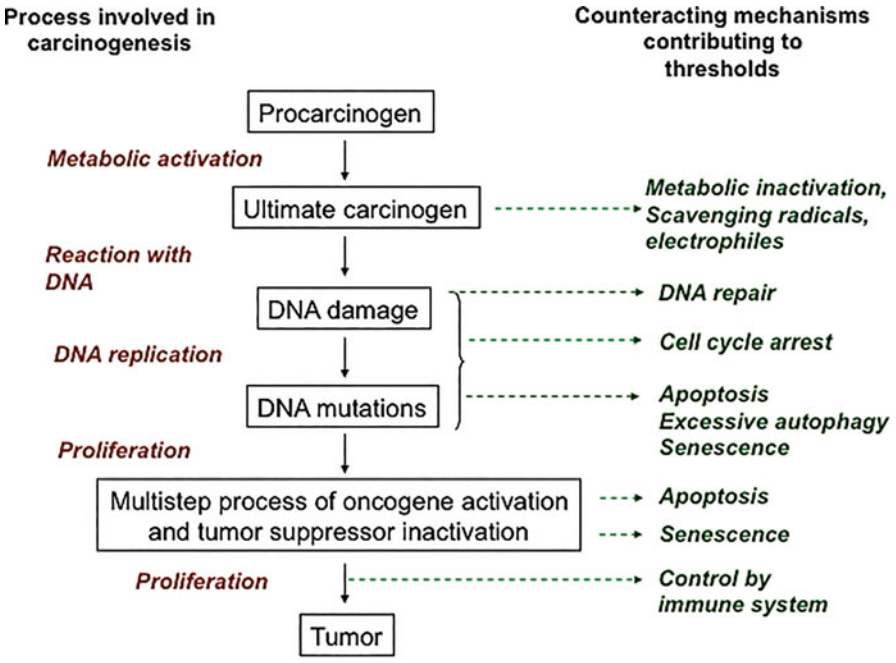

Fig. 1 Multistep process of carcinogenesis and factors that possibly determine thresholds

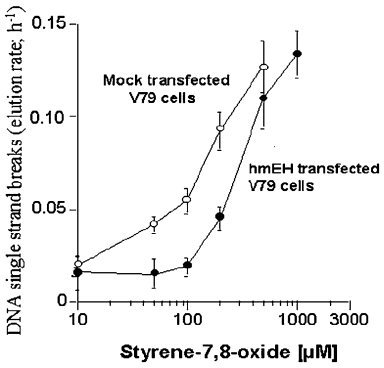

Fig. 2 Expression of recombinant microsomal epoxide hydrolase (*mEH*) protects V79 cells from styrene-7,8-oxide-induced DNA damage. mEH induced a "practical threshold" up to concentrations of 100 µM. (Modified from Herrero et al. 1997)

Carcinogen Detoxification. A typical example is the impact of the microsomal epoxide hydrolase (mEH) on styroloxide-imparted genotoxicity (Fig. 2). Chinese hamster fibroblasts (V79) constitutively express low mEH activity and are, therefore, not able to detoxify styroloxide efficiently. Consequently, a threshold following styroloxide exposure was not observed in this cell line (Herrero et al. 1997). Following transfection and expression of human mEH, V79 cells are able to inactivate styroloxide by cleaving a reactive epoxide group in the molecule, and, therefore, they display a threshold (Fig. 2; in a linear plot a hockey stick curve). In human cells, the involvement of a radical scavenger, glutathione, has been implicated in a no-effect threshold upon treatment with hydrogen peroxide (Seager et al. 2012). On the other hand, theoretical examinations render a model unlikely in which detoxifying enzymes work "perfectly." In fact, it is likely that some molecules of a carcinogen escape detoxification and thus induce DNA damage at low doses. Thus, we assume that detoxifying enzymes can cause a "practical" but not a genuine no-effect threshold.

DNA Repair. DNA repair mechanisms are potentially the main causes of no-effect thresholds. In this context, we assume that DNA repair of premutagenic adducts prior to replication is free from errors. Of note is the protection offered by the suicide repair protein O^6-methylguanine-DNA methyltransferase (MGMT) in the repair of the critical premutagenic adducts O^6-methylguanine (O^6MeG) and O^4-methylthymine (O^4MeT) and, thus, in the prevention of point mutations. MGMT is also very efficient in protecting against MNU and chloroethylnitrosourea-induced skin cancer formation by blocking the tumor initiation, but not the tumor promotion step (Becker et al. 2003). It is important to note that overexpression of MGMT significantly protected, but not completely nullified, the effect of MNU in tumor initiation (Becker et al. 2014). We should consider the possibility of mispairing mutations at replication forks, which may occur in replicating cells before the lesion is removed. In contrast to overexpression, lack of MGMT renders mice highly susceptible to colon cancer formation induced by azoxymethane (AOM), an O^6-methylating colonotropic agent (Ochiai et al. 2001; Wirtz et al. 2010). Comparing colon cancer formation (using the elegant technique of mini-colonoscopy) upon

AOM initiation and sodium dextran sulfate mediated promotion in MGMT and alkyladenine -DNA glycosylase (AAG)-deficient mice, which are base excision repair (BER) defective, it turned out that MGMT, but not AAG, causes a threshold (Fahrer et al. 2015). This finding can be explained by the minor role of N-methylpurines in the induction of point mutations.

Even more complicated is the situation if we consider the susceptibility of PARP-1 knockout mice. PARP-1 (poly(ADP-ribose) polymerase-1) participates in several DNA repair pathways, including BER. PARP-1 knockouts (ko) showed a lower tumor incidence than wild-type mice, whereas MGMT/PARP-1 double ko developed more but smaller tumors than MGMT ko animals. The findings revealed PARP-1 as a double-edged sword in colorectal carcinogenesis: it suppresses tumor initiation following DNA alkylation in a MGMT-dependent manner, but promotes inflammation-driven tumor progression (Dorsam et al. 2018). This unexpected observation shows how difficult it is to arrive at conclusions regarding thresholds just from theoretical considerations. Nevertheless, there is a bulk of data supporting the notion that MGMT is at the forefront in setting up thresholds in organs that are targeted by O^6-methylating carcinogens (i.e., agents inducing O^6methylguanine). Data has also shown the importance of MGMT-mediated repair of O^6MeG in the no-effect level of MNU-induced point mutations in lymphoma cells (Johnson et al. 2012; Thomas et al. 2013). Once this protection has been removed, the threshold dose is reduced (Zair et al. 2011).

We wish to stress the point that upregulation of DNA repair genes or repair activity following genotoxic stress would allow the cell to tolerate a higher dose of chemical and should potentiate a no-effect threshold. This was first shown in *E. coli*, in which MNNG treatment induces the expression of the *ada* gene, thereby equipping the cell with significantly more Ada protein (alkyltransferases) as part of the "adaptive response." Upon future exposures, adapted cells tolerated a higher exposure without an increase in mutation frequency (Lindahl et al. 1988). While the *MGMT* gene has been shown to be inducible in rodent hepatocytes, it was not found to be upregulated in human fibroblasts (Fritz et al. 1991). A recent comparative study with human cancer cells revealed that MGMT is not really subject to upregulation by ionizing radiation and DNA alkylation through temozolomide, but there was to some extent significant increase in the MGMT level following glucocorticoid treatment (Aasland et al. 2018). Therefore, hormone and growth factor regulation should also be taken into account if thresholds are discussed.

In human cells, the nucleotide excision repair (NER) genes *XPC* and *DDB2* are upregulated in response to p53 stabilization following genotoxic stress, whereas the *XPF* and *XPG* NER genes are upregulated via the transcription factor AP-1 (Christmann et al. 2006) following exposure to ultraviolet light (UV). This induction protected the cells against a second, "challenging" dose of UV and, thus, provides an example of a genuine adaptive response in mammalian cells (Tomicic et al. 2011). Similar upregulation was also observed for apurinic endonuclease as part of the adaptive response to oxidative stress (Ramana et al. 1998). On the contrary, there are indications that increased repair activity does not necessarily confer greater resistance to cell death and mutation. DNA mismatch repair (MMR), base excision repair

(BER), and NER are complex pathways involving the coordinated activity of sequentially working enzymes. Overexpression of only one enzyme in the pathway can lead to unbalanced DNA repair, conferring a hypermutable phenotype, which was shown for cells overexpressing *N*-methylpurine- DNA glycosylase (MPG), a primary glycosylase in the BER pathway that removes *N*-alkylpurines from DNA (Coquerelle et al. 1995).

Additionally, some DNA polymerases involved in translesion synthesis (TLS) are known to be error-prone and induce mutations as they bypass bulky lesions to prevent replication fork stalling. One of these is DNA polymerase eta (POLH), which is upregulated on gene level following exposure of cells to benzo(a)pyrene diol epoxide (BPDE) and other genotoxicants that activate p53. As a consequence, BPDE exposure gives rise to genotoxic tolerance in the sense that survival of cells is enhanced at the expense of mutations (Christmann et al. 2016). Whether this has impact on cancer initiation and progression remains an open question. Theoretically, genotoxic tolerance (i.e., tolerance of replication-blocking lesions) could be reduced by a lower expression of these "error-prone" polymerases, or the adduct is removed from DNA before it interferes with replication by error-free NER.

It could be said that post-replicative DNA repair of base mispairs could contribute to a no-effect threshold level. However, in the example of post-replicative MMR processing of O^6MeG-thymine mispairs, the outcome can be unpredictable. In this case, although thymine is removed, it is reintroduced due to the miscoding potential of O^6MeG. An ensuing futile cycle eventually leads to DNA strand breaks that are toxic to the cell. This mechanism is thought to remove cells from the cell pool, which harbor mutagenic O^6MeG adducts, which has in fact been suggested to occur in colon carcinogenesis induced by azoxymethane (Wirtz et al. 2010). O^6MeG is a highly mutagenic, clastogenic, and recombinogenic lesion (Kaina et al. 1993). Therefore, MMR-driven cell killing may be considered a causal factor for causing a no-effect threshold for gene mutations, clastogenicity, and cancer formation.

DNA Damage Response. The repair of adducts by such excision repair processes has been the sole focus of mechanistic studies into the threshold-dose responses observed. However, many different types of adducts can result in clastogenic DNA double-strand breaks (DSBs) and become substrates for the DNA damage response (DDR). The influence of the DDR in a cancer threshold may be twofold. Firstly, at the DNA level, DDR contributes to the cellular tolerance to the possible damage from adducts via its role in the orchestrated repair of DSBs following cell cycle arrest and homologous recombination (HR) or non-homologous end-joining (NHEJ), assuming faithful repair. One would imagine this would be a similar situation to adduct repair, which has already been substantiated in thresholds. Secondly, at the cellular level, the DDR plays an important role in determining different cell fates, following treatment (Roos et al. 2016). In this regard, the DDR removes or immobilizes damaged cells via apoptosis and senescence, respectively. Indeed many cancers display mutations in key DDR enzymes, and, furthermore, many cancer predisposition syndromes stem from inherited defects in DDR components such as ATM, ATR, and p53. Therefore, its role as a defense mechanism in carcinogenesis is

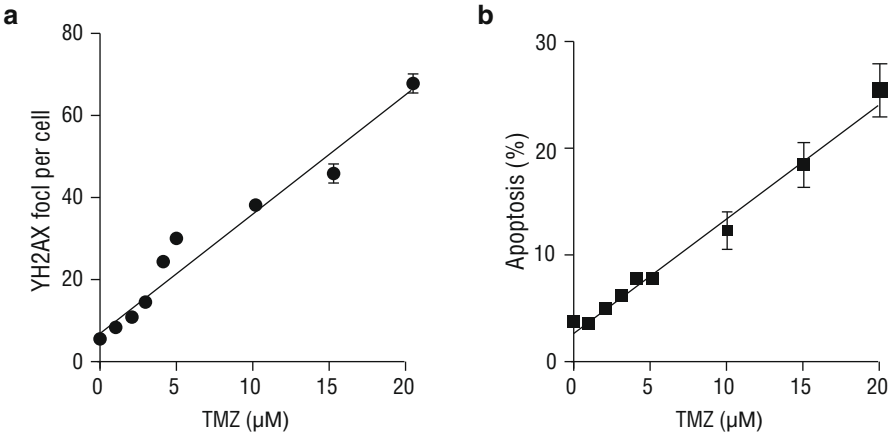

Fig. 3 Dose response of LN-229 glioblastoma cells treated with temozolomide. (**a**) γH2AX foci and (**b**) apoptosis were measured 48 and 96 h after treatment with TMZ, respectively. (Modified from He and Kaina 2019)

realized. It is hypothesized, and textbook knowledge, that at low doses, the DDR arrests cells in the cell cycle, giving time for repair of the damage, while at high doses, senescence or apoptosis (which may have its own threshold of damage) is induced. However, this classical concept has been challenged by the observation that the dose- response of phosphorylated H2AX (γ-H2AX) is a linear function of dose (Fig. 3a) and activation of HIPK2, the kinase responsible for activating the death pathway (He et al. 2019), increases with a similar linear function at the posttreatment time of analysis (He and Kaina 2019). Although the data were obtained on cancer cells treated with the alkylating drug temozolomide, there are indications that γ-H2AX is a linear function of dose/concentration in other cell systems as well (B. Kaina, unpublished data). Nevertheless, it can be concluded that DDR plays a pivotal role in protecting against cancer and reducing the risk of exposure, which has been discussed extensively especially for simple alkylating agents (Thomas 2020).

Apoptosis. The process of programmed cell death (apoptosis) is generally seen as a process to remove mutated cells. It is reasonable to posit that elimination of cells harboring critical mutagenic adducts or, at a later stage, cells in which these adducts have caused mutations in critical genes, counteracts tumor initiation and tumor progression. The existence of "sensor mechanisms" is postulated to trigger the apoptotic pathway in response to DNA damage. The critical lesion O^6MeG provides us with a useful example: it is highly mutagenic, pre-carcinogenic, and a potent trigger of apoptosis. Signaling studies have shown that the MMR proteins MSH2 and MSH6 initiate apoptosis through a variety of pathways upon recognition of O^6MeG-thymine mispairs (Quiros et al. 2010).

It is often speculated that only severely damaged cells are removed from the population through cytotoxicity occurring at high doses. At low doses, however, with comparatively low levels of DNA damage, the trigger for apoptosis may be

insufficient, and therefore, apoptosis may not play a role in low-dose thresholds. In this "classical" model of cell death through apoptosis, p53 plays a key role as switch maker between survival and death. In more detail, the model claims that critical DNA lesions that block replication, or DSB themselves, activate the DNA damage-responsive kinases ATM and ATR, which in turn phosphorylate SIAH1, an inhibitor of the kinase HIPK2. This results in activation of HIPK2, which phosphorylates p53 at serine 46 (p-p53ser46) (He et al. 2019). This p53 modification causes p53 to act as a tetrameric transcription factor for pro-apoptotic genes, such as *BAX* and the death receptor *Fas*. In this model, p-p53ser46 serves as stimulator of pro-apoptotic genes, while another modification of p53, p-p53ser15, triggers upregulation of anti-apoptotic genes, including DNA repair genes such as *DDB2* and *XPC* (Christmann and Kaina 2013). According to this model, it is anticipated that only high doses of genotoxic agents trigger upregulation of the ATM/ATR-HIPK2-p53ser46 axis, but surprisingly (as outlined above) recent experiments revealed a linear, threshold-lacking dose response at the posttreatment time of analysis. Thus, the induction of HIPK2 triggered by O^6MeG was a linear function of dose, and apoptosis did not display a threshold in p53 wild-type cancer cells that are MGMT deficient (Fig. 3b) (He and Kaina 2019). Given that damaged cells become eliminated at low- and high-dose levels, apoptosis is even a more powerful mechanism of removal of precancerous cells than hitherto thought.

We should stress the point that the presence of a no-effect threshold depends on the genotoxic agent and the repair capacity of the cell to be considered. Thus, agents inducing O6-alkylguanine display a clear threshold if cells are MGMT proficient, due to fast and error-free repair of the killing (and mutagenic) lesion, while in MGMT-deficient cell types, a threshold may be lacking (as outlined above). Genotoxicants that induce bulky lesions or induce DNA breaks via inhibition of topoisomerases may show a threshold due to activation of HIPK2 death pathway, which was shown to occur in this case only at high-dose levels (Winter et al. 2008).

Apoptosis is a critical eventuality of response following exposure, but there are many other outcomes for a cell (senescence, autophagy, mitotic catastrophe, necroptosis, ferroptosis, and others), and the importance of dose in determining cell fate is yet to be fully elucidated. Nevertheless, the fate of a cell, following genotoxic exposure, may play a crucial role in likelihood of cancer. Understanding what determines cell fate is of critical importance for its extrapolation into cancer risk.

Dose, Time, and Cell Fate Heterogeneity

While genotoxicity assessment, in terms of mutagenicity and clastogenicity, provides the mainstay of assessing DNA damage tolerance, the ultimate fate of a cell, following treatment, underpins pragmatic dose-response assessment. Cell fates can be broadly classified into distinct phenotypes including cell survival with or without mutations, cell death, cell cycle arrest, and senescence. It is important to note that these outcomes may not all be mutually exclusive but rather successive states that

may culminate in the same cell fate. In this context, the rapid or delayed initiation of a particular cell fate, or the presence or absence thereof at specific times following genotoxic insult, may indicate inherent thresholds, but whether these are dosage thresholds is uncertain, but may be dependent on time. The elucidation of the factors involved in determining cell fate will play a very important role in determining biological risk.

It was shown that human bone osteosarcoma cells, treated with the platinum DNA strand cross-linker, cisplatin (0.07 and 0.14 μM), had nearly indistinguishable growth curves (measured every 30 min until 5 days after treatment) compared to untreated controls (Granada et al. 2020). Moreover, the authors demonstrated distinct cell fates in response to variable cisplatin doses (7, 10, and 13 μM). Of note here is that the reported "medium" dose (10 μM) is not much less than the "high" dose (13 μM), yet these doses elicited distinct cell fates. However, cell fate was also tied to the proliferative index of individual cells preceding treatment. Specifically, 7 and 10 μM doses preferentially caused cell cycle arrest in cells with a high proliferative index (HPI). Surprisingly, cells of low proliferative index (LPI) preferentially died. One would imagine that LPI cells would have fewer mutations than those in the same culture with HPI. However, mutation induction was not measured in cells of either status. Nevertheless, the study highlights that cell fate, even within a population exposed to the same insult, is heterogeneous and dependent upon biological factors other than dose. The impact of dose on cell fate is, therefore, difficult to predict. However, when examining the relationship in reverse, i.e., the impact of cell fate on eliciting a genotoxic threshold, the death of cells with low proliferative index may be a contributing factor.

Exploring this a little further, it is not unreasonable to assume that certain cell populations may respond heterogeneously to DNA damage, as a result of having different transcriptomes. This could differ between cell types and, as the previously mentioned study suggested, even across individual cells within an experimental system both in vitro and in vivo over time, to facilitate specific functions, i.e., cell division as previously eluded to. It is possible that the transcriptomes of some cells are geared toward a lower genotoxic threshold of apoptosis and perhaps a higher threshold level for cancer. For example, mouse epiblasts are more susceptible to undergo apoptosis following low-dose irradiation than surrounding tissue (Laurent and Blasi 2015). The authors demonstrated pronounced 53BP1 accumulation at DSB sites within 10 min of 0.5 Gy X-ray irradiation in epiblasts, but not visceral endoderm (VE) or extra-embryonic ectoderm (ExE) cells. Similarly, p-p53ser18 phosphorylation was readily observed within the same time and dose in epiblasts but only after 1 h in ExE cells. The authors alluded to epiblasts being primed for apoptosis as evidenced by inherently elevated levels of pro-apoptotic Bim, Bak, and Noxa and decreased levels of anti-apoptotic Bcl-xL, as compared to extra-embryonic tissues. These findings underpin the ability of certain tissues to have an inherently lower apoptosis threshold to prevent mutation propagation. On the other hand, Paek et al. (2016) demonstrated the apoptotic threshold to increase with time following cisplatin treatment, owing to increased abundance of inhibitors of apoptosis. Therefore, it seems reasonable to infer that in a particular cell, the DNA damage threshold to elicit cell apoptosis is dynamic and may be shifted by intrinsic or extrinsic mechanisms.

In line with this notion, non-coding RNAs may function as intrinsic modulators of the DNA damage threshold. Specifically, small non-coding RNAs such as microRNAs (miRNAs) are promising candidates as they are known to regulate several DNA repair genes (reviewed by Wang and Taniguchi 2013). Consequently, as DNA repair mechanisms are potentially key determinants of no-effect thresholds (reviewed by Jenkins et al. 2010) and cell fate, the importance of miRNAs is evident. For example, miR-192 hinders NER in liver carcinoma cells. miR-192 directly targets ERCC3/4, components of NER (Xie et al. 2011). Moreover, poly (ADP-ribose) polymerase (PARP1) is a proposed target of miR-7-5p (Luo et al. 2018; Lai et al. 2019). Importantly, PARP1 functions in several DNA repair pathways including NER (Robu et al. 2013) and base excision repair (BER) (Reynolds et al. 2015) and modulates homologous recombination repair (HRR) (Lai et al. 2019). Lai et al. (2019) demonstrated endogenous miR-7-5p expression to be significantly reduced in doxorubicin (dox)-resistant small-cell lung cancer cells (SCLC), compared to dox-responsive SCLC cells. Moreover, miR-7-5p transfection significantly reduced the dox IC_{50} in dox-resistant SCLCs. Luo et al. (2018) demonstrated that lymphoblastoid cell transfection with miR-7-5p, followed by hydroquinone treatment, resulted in a significant increase in apoptosis and, hence, a lower damage threshold. Likewise, miR-7-5p transfection also prominently increased the proportion of apoptotic cells both with and without hydroquinone treatment. This suggests miR-7-5p-mediated PARP1 inhibition is sufficient to drive the DNA damage threshold below endogenous DNA damage levels. As PARP1 was implicated as a target for suppression by miR-7-5p in both the study by Luo et al. (2018) and Lai et al. (2019), PARP1 inhibition appears to be the principle pro-apoptotic and anti-survival mechanism of miR-7-5p. By extension, this also implies a mechanism for reduced DNA damage tolerance, especially considering the consequences of PARP1 inhibition likely stem from its impaired role in multiple DNA repair mechanisms. These examples exemplify the potential for miRNAs to shift DNA damage thresholds and, at least partially, promote a pro-apoptotic cell fate. Whether they contribute to a cancer threshold is only postulated, but warrants further investigation.

Immune System. The immune system is equipped with cells to recognize and eliminate tumor cells. The system involves dendritic cells that may represent tumor cell antigens, cytotoxic T cells, neutrophils, and macrophages that respond upon activation with a cytotoxic ROS burst. The question remains if tumor cells are targeted following initiation, but prior to phenotypic transformation. It can only be speculated that a no-effect threshold would be dependent upon a perfectly functioning immune system, which becomes saturated due to an increased demand for removal of tumor cells at higher concentrations, where more cells are initiated. The role of immunity against genotoxicant-initiated cancer cells in thresholds has not yet been addressed experimentally. But there is growing evidence that the immune system can become activated to eradicate cancer cells. Impressive examples are checkpoint inhibitors (Chapman et al. 2011) and immunomodulators (Hogan et al. 2018), which abrogate the inhibitory activity of CTLA-4 cytotoxic T-cell receptor and tumor T-cell ligand interactions through PD-1 inhibition, respectively,

thus activating cytotoxic T cells to target and destroy tumor cells. Whether the CTLA-4 and PD-1 pathways are subject of genotoxicant mediated regulation is an open and highly challenging question.

Further Examples of Genotoxic Carcinogens

The following examples outline the heterogeneity of the dose-response relationship among direct acting genotoxicants. For most genotoxic carcinogens, the linear assumption holds true. These include aflatoxin (B1), diethylnitrosamine (DEN), and the tobacco-specific carcinogen 4-(*N*-methyl- *N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Figure 4a-c shows their dose-response relationship for tumorigenesis and the amount of adducts involved. The largest tumorigenesis research was performed with 2-aminoacetylfluorene (2-AAF). In a large study (24,000 mice), researchers were unable to prove a threshold for the induction of liver tumors (Littlefield et al. 1980) (Fig. 4d).

Despite these definitive examples, we should not assume the generalizability of the linear model for all genotoxicants given recent and ever-increasing support for nonlinearity. For example, tumor induction by vinyl acetate has a no-effect level of $<$100 mg/kg/day (Fig. 5). The underlying mechanism has been exhaustively discussed (Hengstler et al. 2003).

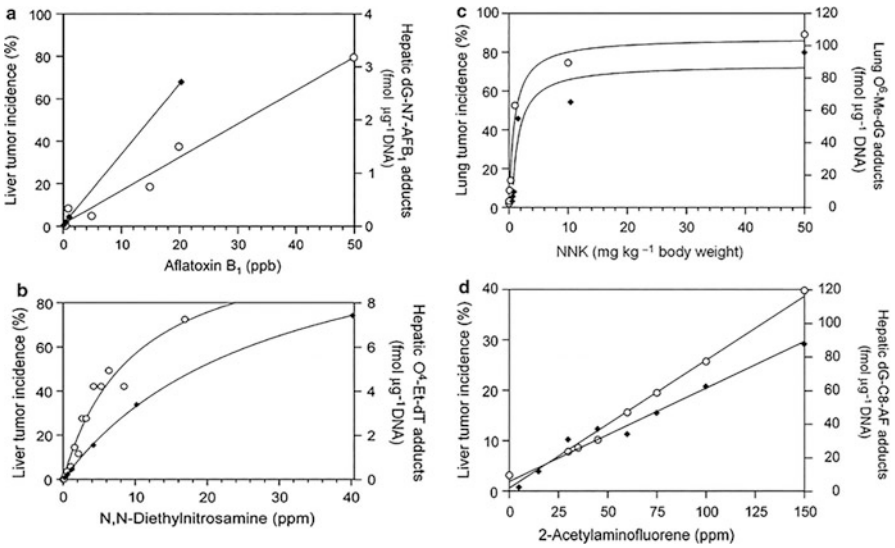

Fig. 4 Induction of tumors (o) and DNA adducts (♦) induced by different carcinogens as a function of dose. (**a**) Incidence of liver tumors and aflatoxin B1 adducts in rat livers. (**b**) Induction of liver tumors in rats by DEN. Incidence of liver tumors (o) and the DNA adduct O ^{4}-ethylthymine (♦) is shown as a function of dose. (**c**) Incidence of lung tumors (o) and levels of O ^{6}-methylguanine (♦) in rats after administration of NNK. (**d**) Incidence of liver tumors (o) and DNA adducts (♦) after administration of 2-acetylaminofluorene to BALB/c mice. (Data are from publications compiled in Hengstler et al. 2003)

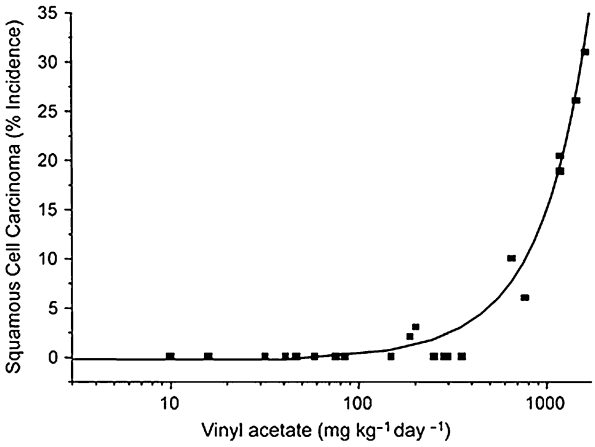

Fig. 5 Incidence of squamous cell carcinoma as a function of vinyl acetate dose in rodents. (From Hengstler et al. 2003)

Regulatory Aspects on Thresholds or No Thresholds for Genotoxic Carcinogens

The most important differentiation for genotoxicants in the regulatory context today is its reactivity with the DNA. Examples of mechanisms of genotoxicity that are considered to lead to nonlinear or thresholded dose response have been listed in the European "Guideline on the Limits of Genotoxic Impurities" published by the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMA) in 2006 (CHMP 2006). The examples listed there comprise genotoxic mechanisms like interacting with the spindle apparatus of cell division leading to aneuploidy, topoisomerase inhibitors, inhibition of DNA synthesis, overloading of defense mechanisms, metabolic overload, and physiological perturbations (CHMP 2006). Guérard et al. (2015) describe the impact of such mechanisms for the genotoxicity of oxidants, topoisomerase inhibitors, and aneugens and also the impact of metabolism and detoxification for nonlinear dose response of genotoxicants. For genotoxic compounds with clear evidence for acting via such a thresholded mechanism, determination of a threshold dose is considered acceptable (CHMP 2006).

For genotoxicants acting by direct chemical reaction with DNA (DNA-reactive), determination of a threshold dose defining a no-risk level is considered extremely difficult. As stated in the introduction, answering the question of a threshold for such compounds will require extensive work. Nevertheless in regulation of chemical substances, acceptable limits for daily exposure are defined. These limits are defined by various methods of modeling the dose response in animal carcinogenicity studies to determine a point of departure (PoD) for a linear extrapolation to the 0-point. Depending on the area, different PoDs like TD_{50} or $BMDL_{10}$ and different adjusting factors are used to determine the dose associated with a risk level or a margin of

exposure considered acceptable. The risk levels considered acceptable (e.g., $\leq$ 1: 100,000) (ICH 2017) are so low that experimental proof in life time animal carcinogenicity experiments is simply not possible as this would require millions of animals for one experiment. Therefore, the risk levels are referred to as a "theoretical cancer risk."

In our daily life, we are exposed not only to one genotoxic compound or one tumor promoter, but many. Cooked or fried food, combustion exhausts, cured meat, active and passive smoking, or natural plant products contain a considerable number of mutagenic carcinogens, tumor promoters, and compounds interfering with carcinogen activation and detoxification. Determination of a threshold for a certain DNA-reactive chemical would need deciphering all the key steps it takes to cause cancer from transport into the cell, reacting with DNA, forming adducts, steps needed for transformation into mutation, hitting all necessary genes needed for cellular transformation, and bypassing apoptotic mechanisms and immune defense. For all steps, a threshold dose needs to be determined and finally an overall threshold by modeling the whole process for this chemical. Different people have different exposure to other chemicals interfering with the activation, detoxification, and defense mechanisms. Individuals also differ by their individual capacity for each step due to, e.g., genetic variations in key enzymes. The threshold must therefore be transferred into a limit by applying adjustment factors to be protective for a heterogeneous population. To protect a population, it can be argued that the threshold for the first critical step, the reaction with DNA, will provide a protective threshold. This however requires to measure this threshold reliably in experiments, which is difficult and may only be possible in cases where DNA adducts can be reliably distinguished from the background of endogenous adducts. Actually, there are examples showing that this can be achieved. Thus, the difference in the dose response of MGMT lacking and MGMT expressing cells is dramatic if O^6MeG following treatment with an alkylating agent is measured (Kraus et al. 2019). This also leaves out all defense processes of cells and organisms and may lead to a very low threshold with questionable biological relevance. Standard studies to detect gene mutations in mammalian cells and animals are used for regulatory purposes to verify or deny the hazard of increased mutation formation. The data are not used to quantitatively analyze the dose-response relationship and derive PoDs used for extrapolations and risk assessment to determine regulatory limits. One major problem for determining a threshold in these assays is the impossibility to distinguish the mutations caused by the chemical from the background mutations. The formation of mutations is one key step in the development of cancer by genotoxic chemicals. It is considered plausible that dose levels not increasing mutation frequency also will not lead to an increase in cancer. It may therefore be possible to use dose-response modeling to determine a PoD in mutation assays which can be used to extrapolate protective limits. Major investigations are ongoing, and an example how this may be done is described for benzo[a]pyrene by Long et al. (2018).

The relationship between dose responses in adduct formation, mutation frequency, and increase in pre-carcinogenic and carcinogenic lesions in organs is described in MacGregor et al. (2015) for the mutagenic carcinogen

2-amino-3,8-dimethylimidazol[4,5-f]quinoxaline (MeIQx), one of the still very rare examples such data are available for. In this example, the BMD for 10% increase over background (BMD_{10}) is used for comparison. BMD_{10} and $BMDL_{10}$, the lower end of its 90% confidence interval, are the most frequently used in comparison of results in rodent carcinogenicity studies. The BMD_{10} is increasing from adduct formation to cancer in rat by four to five orders of magnitude. Such data suggest that below a certain dose level where the risk for genetic damage is small, the subsequent risk for developing cancer is negligible. This comparison supports the hypothesis that a threshold for MeIQx may eventually exist, although it cannot be determined yet. As outlined above, a threshold for O^6MeG resulting from its repair by MGMT (Kraus et al. 2019) and mutations and cancer resulting from this damage has been demonstrated unequivocally (see above). Whether these findings can be translated to humans expressing different amounts of repair enzymes is an open question.

In contrast to O^6-methylating genotoxicants and MeIQx, the data above shown for DEN, NNK, AFB1, and 2-AAF do not seem to support the hypothesis that no-effect thresholds for carcinogens are the rule. Of note, O^6-ethylguanine is removed from DNA less efficiently than O^6MeG, which may cause a difference in responses and possibly also thresholds elicited by DEN versus DMNA. There are presumably more exceptions, and the examples given here demonstrate that the potencies of mutagenic carcinogens vary widely. For the highly potent carcinogens (except the O^6-methylating agents), a threshold may be extremely low and in a dose range simply impossible to measure in any assay. At least the biological mechanisms behind chemical carcinogenesis are the same.

The bottleneck is still the experimental proof of a no-effect threshold or even a practical threshold with no biologically relevant effect for mutagenic carcinogens. Extensive scientific investigations are ongoing. Until there is experimenal evidence for the existence of “genuine” thresholds, the need of regulatory toxicology for setting acceptable limits for mutagenic carcinogens has to be covered by the established methods at hand.

Conclusions

A practical no observed effect level could occur with genotoxic carcinogens. Effective detoxification, scavenging mechanisms, and lack of activation could prevent DNA interaction. Critical DNA adducts are subject to error-free repair, which might be considered a major mechanism provoking a no-effect threshold. Furthermore, elimination of damaged cells by apoptosis and premalignant cells by the immune system may further reduce the level of risk. The concept of a threshold is theoretically plausible at each requisite step involved in chemical carcinogenesis. Manipulating the intracellular levels of metabolizing enzymes and upregulation of MGMT and other repair enzymes in a balanced fashion, as well as increase in their activity following genotoxic stress through post-translational mechanisms, may potentiate the existence of a threshold. Conversely, due to the complexity of the DNA repair and damage response pathways, altering expression of effector proteins may

unbalance the repair process and abrogate a threshold by promoting genomic instability and mutagenesis. The processes discussed here have been implicated as the casual event in nonlinear dose-response relationships. Whether the observed thresholds are a true null effect remains to be seen. Mechanistic investigations are ongoing to discover the biological relevance of low-dose exposures. Such studies are mandatory in the acceptance of no-effect thresholds.

Cross-References

- ▶ Benchmark Dose Approach in Regulatory Toxicology
- ▶ Dose-Response Analysis: Identification of Threshold Levels for Chemicals
- ▶ Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds
- ▶ Health Hazard Classification and Labelling
- ▶ Integration of Advanced Technologies into Regulatory Toxicology
- ▶ Specific Toxicity Tests for Neurotoxicity, Immunotoxicity, Allergy, Irritation, Reprotoxicity, and Carcinogenicity
- ▶ The Regulatory Process in Toxicology
- ▶ Toxicity Testing In Vitro: Regulatory Aspects

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Single Substances Versus Combined Effects: Problems in Mixture Toxicology

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Thomas R. H. Büch, Eva A. M. Büch, Ingrid Boekhoff, John H. Duffus, and Thomas Gudermann

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Abstract

Effective protection against harmful effects of toxic substance mixtures requires the ability to assess the combined risk potential of the various constituents. The biological impact of chemical mixtures may arise from independent, additive, synergistic, or antagonistic effects of single constituents. Mathematical models may be used to characterize corresponding toxic effects. In most cases, models act

T. R. H. Büch
Rudolf Boehm-Institute for Pharmacology and Toxicology, Clinical Pharmacology, Leipzig University, Leipzig, Germany
e-mail: thomas.buech@medizin.uni-leipzig.de

E. A. M. Büch
Leipzig University Cancer Center (UCCL), University of Leipzig Medical Center, Leipzig, Germany
e-mail: eva.buech@medizin.uni-leipzig.de

I. Boekhoff · T. Gudermann (✉)
Walther Straub Institute of Pharmacology and Toxicology, Ludwig-Maximilians-Universität München, Munich, Germany
e-mail: ingrid.boekhoff@lrz.uni-muenchen.de; thomas.gudermann@lrz.uni-muenchen.de

J. H. Duffus
Toxicology, The Edinburgh Centre of Toxicology, Edinburgh, Scotland, UK
e-mail: J.H.Duffus@blueyonder.co.uk

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on the assumption of independent effects or unknown mechanisms of action. However, a mechanistic understanding of interaction/s among mixture constituents, if available, is the best basis for quantitative predictions of the consequences of co-exposure to different stressors. In the following chapter, the possible modes of interaction of substance mixtures and the general mechanisms of interactions are described. Furthermore, formulae to quantify the combined risk of co-exposure to multiple substances are depicted.

Keywords

Risk potential · Agonist · Antagonist · Agent-to-agent interaction · Mixture toxicology · Hazard quotient · Hazard index

Introduction

It has long been known that co-exposure to different substances may trigger effects other than the simple summation of the effects caused by the individual constituents. The supra-multiplicative increase in the risk of esophageal cancer by co-exposure to tobacco smoke and ethanol is a well-known example. Nevertheless, most risk assessments performed by regulatory boards like the US Environmental Protection Agency (EPA), the US Food and Drug Administration (FDA), the European Chemicals Agency (ECHA), or the European Food Safety Authority (EFSA) have focused on single substances.

In the following review, the modes of interaction between individual contaminants, including cases of synergism and antagonism, are described. In addition, important concepts for assessing the risks of a combined exposure, such as formulae allowing to quantify the combined risk of co-exposure to multiple substances, are introduced.

Basic Considerations

Multiple substances, acting simultaneously on the human or animal organism, in general result in two fundamental possibilities concerning their toxic effects:

1. The individual substances affect each other neither directly nor indirectly and exert completely independent effects on the exposed organism (independent effects, no interference). Thus, the constituents of the mixture act as if each one was the sole substance in the body.
2. The toxic substances influence each other either via substance-substance interactions (e.g., substance “a” binds and “neutralizes” substance “b”) or via identical toxicological endpoints (e.g., substance “a” and substance “b” target the same organ).

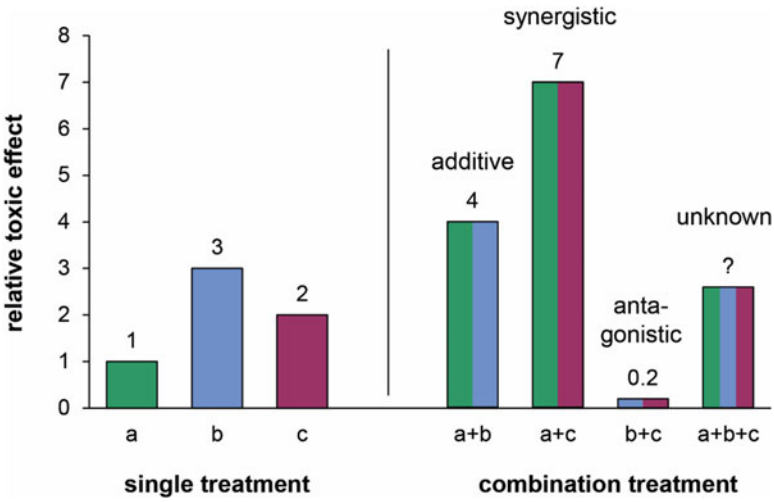

Fig. 1 Combination effects of three single compounds with identical toxicity endpoints. The left columns show the relative toxicity of compound b and c in comparison to the exposure of substance a. Each compound is given as individual substance (*single treatment*). The right group of columns illustrates examples where a simultaneous exposure of two or even all three compounds elicits either additive (a + b), synergistic (a + c), antagonistic (b + c) or not predictable toxic effects (a + b + c) (*combination treatment*). The numbers above the bars in both groups quantitate the relative toxic effect in comparison to substance a. (Modified from Bolt 2004)

In scenario (1), the risk assessment of the substance mixture relies on the analysis of the single compounds.

In scenario (2), a mechanistic understanding of the interaction is desirable for risk evaluation although this is often not available. In such a complex situation, three possibilities are conceivable regarding the resulting combination effects (see also Fig. 1).

- The effects of a substance mixture correspond to the sum of the effects of the single constituents, thus leading to dose additivity.
- The effect of a substance can be increased by another substance. This process results in more-than-additive effects and is known as synergy.
- The effect of a substance is attenuated by the presence of a second substance (antagonism). One utilizes this phenomenon in the therapy of intoxications. The mechanisms behind antagonistic effects are physical, chemical, or biological processes.

In principle, three types of interactions can lead to antagonistic, additive, or synergistic effects of combined substances.

- The single compounds of the mixture can react with each other (agent-to-agent interaction) prior to incorporation or after ingestion in the body. An example of a

reaction occurring outside the body is the formation of ozone and peroxyacyl nitrates as so-called secondary pollutants in the air, following the interaction of hydrocarbons with nitrogen oxides (NOx) in the presence of ultraviolet light. Agent-to-agent interactions occurring within the body have rarely been identified in mixture toxicology. However, this phenomenon is commonly used to neutralize drugs or toxic substances. One example is the binding of excessive muscle relaxants of the aminosteroid type by modified γ-cyclodextrin (sugammadex) or the neutralization of digoxin by application of digoxin-specific antibody fragments.

- A much more common mechanism in mixture toxicology occurs when constituents of the mixture display toxicokinetic interactions. These may lead to altered concentrations of substances in target organs by effects on the elimination or distribution. An illustrative example of a toxicokinetic interaction is the consumption of ayahuasca, a hallucinogenic mixture of *Banisteriopsis caapi* vine and *Psychotria viridis* shrubs. The latter ingredient contains serotonergic dimethyltryptamine, and the former contains a monoamine oxidase inhibitor preventing the inactivation of dimethyltryptamine.
- Another very important mechanism of altered toxicity of substance combinations relies on toxicodynamic interactions. This means that the effect of a substance on a target structure is altered by another substance that in turn modifies the susceptibility of the target structure. Many well-established examples for toxicodynamic interactions are related to the field of carcinogenesis. For instance, the methylation of DNA by many DNA-methylating carcinogens like methylnitrosourea or dimethyl nitrosamines can be repaired by O^6-methylguannine DNA methyltransferase (MGMT). Thus, co-exposure to inhibitors of MGMT substantially increases the DNA damaging and carcinogenic effects of these methylating agents.

Basic Aspects of the Scientific Evaluation and Regulatory Specifications of Safety Values for Combined Substance Exposures

The regulatory recommendations for risk assessment of substance mixtures suggest the use of empirical toxicity data, if available. Unfortunately, the experimental investigation of substance interactions using a full factorial design (every combination is tested) is limited by the high number of permutations to be tested. Thus, to test the interaction of two substances using three different concentrations of each compound leads to $3^2 = 9$ combinations, whereas the testing of five substances leads already to $3^5 = 243$ combinations. However, most toxicologically relevant mixtures contain far more constituents. For example, cigarette smoke contains over 7.000 gaseous and particulate compounds among them more than 70 identified carcinogens.

In the case that no toxicity data for the substance mixture are available (which is the normal situation), the regulatory recommendations suggest combining the

toxicity data of single constituents in an additive manner. As a rule of thumb, additive effects are most likely in mixtures of compounds with similar modes of action (especially with identical molecular target structures), whereas independent, antagonistic, or synergistic effects may appear if the substances have different modes of action.

To quantify additive effects of substance mixtures, the calculation of the Hazard Index is an appropriate approach. The Hazard Index results from the concentration (C) of the individual substance in the mixture and the reference dose (RfD, for ingestion or transdermal uptake) or the reference concentration (RfC, for inhaled exposures) (see formula below). The RfD (or RfC) value is defined as the highest dose or concentration of an individual constituent that, as an independent exposure, does not produce harmful effects. Another important factor for a single compound is the Hazard Quotient (HQ), which calculates the ratio of the potential exposure to a substance and the level at which no adverse effects are expected.

$$\text{Hazard Index}: \frac{C1}{RfD1} + \frac{C2}{RfD2} + \cdots \frac{Cn}{RfDn} = \sum_{i=1}^{n} HQ_i$$

Hazard Index: A value < 1 indicates that exposure is unlikely to be harmful
HQ_1 = Hazard Quotient of each individual substance

Since the Hazard Index is based on the assumption of an additive mode of action, its application leads to an overestimation of the real risk of the substance mixture if antagonistic effects occur. Vice versa, the Hazard Index will underestimate the risk of a mixture if synergistic effects play a role. An established application of the Hazard Index is evaluating the risk from exposure to hazardous air pollutants.

Cross-References

- ► Assessment of Mixtures: Bridging Principles and Other Approaches
- ► Notification of Hazardous Mixtures and Cosmetic Products for Poisons Centers in the European Union

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Biomolecules Versus Smaller Chemicals in Toxicology: ICH, EU, and US Recommendations

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Abstract

For many life science professionals, biological products represent the cutting edge of medical research and are the smartest means to target and treat a variety of disease and conditions for which the current treatments are still unsatisfactory. In contrast to small molecule drugs, including new chemical entities (NCEs), biomolecules (also

M. Ruthsatz (✉)
Nutrition+HealthCARE, Vevey, Switzerland

C. Chiavaroli · M. A. Cassar
Voisin Consulting Life Sciences, Lausanne, Switzerland
e-mail: chiavaroli@voisinconsulting.com; cassar@voisinconsulting.com

E. M. Voisin
Voisin Consulting Life Sciences, Boulogne, Paris, France
e-mail: voisin@voisinconsulting.com; vcparis@voisinconsulting.com

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called biologics, biologicals, biopharmaceuticals, or biotechnology-derived pharmaceuticals) are complex macromolecules, sometimes occurring as mixtures that are not easily identified or fully characterized. Nevertheless, due to the rapid development of biotechnology in the last three decades, the number of approved biomolecules is increasing at a faster rate than it is the case for new chemical entities. Biologicals are usually highly specific for a target, are more heat sensitive and susceptible to microbial contamination, and are likely antigenic. Thus, the quality and security testing of biologicals is becoming increasingly important. This updated chapter compares the evolving regulatory environment relevant for biomolecules, with a typical "case-by-case" development program versus NCEs, which are generally developed according to a more standard "classical" manner.

Keywords

Chemical entities · Small molecules · Biomolecules · Biologicals · Toxicology

Introduction

Biotechnology-derived pharmaceuticals (biopharmaceuticals, biologicals, or more simply biologics) are defined as products derived from characterized cells through the use of various expression systems including bacteria, yeast, insect, plant, and mammalian cells. The active substances include proteins and peptides, their derivatives, and products of which they are components; they could be derived from cell cultures or produced using recombinant DNA technology including production by transgenic plants and animals.

Conversely, a NCE (new chemical entity) can be defined as a novel drug substance obtained by chemical change or synthesis and not yet approved for the prevention or treatment of human diseases.

In the EU, all human medicines derived from biotechnology and other high-tech processes are evaluated by the European Medicines Agency (EMA) via the centralized procedure (Notice to Applicants 2016, Vol. 2A).

In the USA, the regulatory evaluations are made either by the Center for Biologics Biopharmaceuticals Evaluation and Research (CBER) or by the Center for Drug Evaluation and Research (CDER). The mission of the CBER at the Food and Drug Administration (FDA) is to ensure the safety, purity, potency, and effectiveness of biological products including vaccines, allergenics, blood and blood products, and cells, tissues, and gene therapies for the prevention, diagnosis, and treatment of human diseases, conditions, or injury. As part of the FDA, the Center for Drug Evaluation and Research (CDER) regulates over-the-counter (OTC) and prescription drugs, including biological therapeutics and generic drugs.

In order to reduce regional discrepancies, the International Conference of Harmonization (ICH) has contributed to a significant global standardization of test conditions and regulatory approval of drugs for quality (ICH Q guidelines), safety

(ICH S guidelines), efficacy (ICH E guidelines), and multidisciplinary (ICH M guidelines). ICH guidelines consider current practices from participating countries and provide a unified view, intended to facilitate mutual acceptance of data and clarify key issues. However, they are typically not statutory by definition and hence not usually legally binding or directly enforceable. The aim of this updated review is to clarify the nonclinical and toxicological regulatory differences encountered when developing NCEs or biologicals, not only taking into account ICH perspectives but also considering regional differences between the EU and US regulations.

Biomolecules Versus Smaller Chemicals in Toxicology

ICH Guidelines

The major ICH guidelines and related Questions and Answers (Q&As) applicable for nonclinical development (ICH S and some ICH M guidelines) are listed in Table 1.

All ICH guidelines listed in this document and their corresponding associated files can be freely downloaded on the https://www.ich.org/ website.

In contrast with the development of NCEs, the list comprises a unique guideline, ICH S6(R1), to address the regulatory environment for the nonclinical development of all biologics (see also Baumann 2009 for a "fundamental review" on nonclinical development of biologics). It is crucial to follow the recommendations of ICH S6 (R1) to achieve the three main goals of nonclinical safety evaluation which are to identify (1) an initial safe dose and subsequent dose escalation schemes in humans, (2) potential target organs for toxicity and for the study of whether such toxicity is reversible, and (3) safety parameters for clinical monitoring. Complying with the recommendations of ICH S6(R1) may, however, still be insufficient to fully predict life-threatening adverse events in man, as discussed below.

It is also important to mention that sometimes assessments may vary between authorities. As an example based on our experience, despite the clear mention in ICH S6(R1) that this guidance may also apply to oligonucleotide drugs, oligonucleotides are usually considered as biotechnology-derived pharmaceuticals by the EMA (following ICH S6(R1), while in the US oligonucleotides are rather evaluated as small molecules by the FDA (following ICH M3(R2)). Therefore, the recommendation for oligonucleotides would be to prepare a nonclinical package that would comply to both guidelines for a worldwide drug development and to request a scientific advice meeting with a regulatory agency to discuss the relevance of the nonclinical development program.

The Regulatory Environment to Initiate First-in-Human Studies

Despite the conduct of a nonclinical development plan in line with the ICH regulations, two dramatic clinical cases necessitated the revision of the regulatory environment for First-in-Human trials in the EU.

Table 1 List of ICH guidelines to assess the safety of NCEs and biotechnology-derived pharmaceuticals

ICH Guideline	Topic (date of coming into force)
M3(R2)	Nonclinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals (re-amended in 2009). Q&As to ICH M3(R2)
M7(R1)	Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk (amended in 2017). ICH M7(R2) is in preparation
S1A	The Need for Long-term Rodent Carcinogenicity Studies of Pharmaceuticals (1995). ICH S1 as a whole is under revision. The goal is to define when 2-year rat carcinogenicity studies are mandatory
S1B	Testing for Carcinogenicity of Pharmaceuticals (1997)
S1C(R2)	Dose Selection for Carcinogenicity Studies of Pharmaceuticals (amended in 2008)
S2(R1)	Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use (amended in 2012)
S3A	Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies (1994). Q&As to ICH S3A: Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies. Focus on Microsampling (2017)
S3B	Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies (1994)
S4A	Duration of Chronic Toxicity Testing in Animals: Rodent and Non-rodent Toxicity Testing (1998)
S5(R2)	Detection of Toxicity to Reproduction for Medicinal Products & Toxicity to Male Fertility (re-amended in 2000, a new version, R3, is in preparation)
S6(R1)	Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals (amended in 2011)
S7A	Safety Pharmacology Studies for Human Pharmaceuticals (2000)
S7B	Nonclinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals (2005). Q&As Clinical and Nonclinical Evaluation of QT/QTc Interval Prolongation and Proarrythmic Potential (in preparation 2018)
S8	Immunotoxicity Studies for Human Pharmaceuticals (2005)
S9	Nonclinical Evaluation for Anticancer Pharmaceuticals (2009). Q&As: Nonclinical Evaluation for Anticancer Pharmaceuticals (2018)
S10	Photosafety Evaluation of Pharmaceuticals (2013)
S11	Nonclinical Safety Testing in Support of Development of Paediatric Medicines (2018, step 2b)

1. In 2006, the press (Suntharalingam 2006) reported that failure to select a safe starting dose in humans at the early clinical stage with the CD28 super-agonist monoclonal antibody TGN1412 led to serious toxicity including multi-organ failure in six healthy volunteers. EMA subsequently issued recommendations and a guideline for the safe and rational conduct of clinical trials (EMEA/CHMP/SWP/294648/2007).
   Therefore, since 2007, both the NOAEL dose (No-Observed Adverse Effect Level, which is related to the "toxicological" effects of a drug) and MABEL dose (Minimum Anticipated Biological Effect Level, which reflects rather the "pharmacological" effect of the drug) should be determined by the Sponsor. The lower of these two

doses should be considered for selecting the starting dose in humans. Even if the European guideline was implemented for both NCEs and biologics, the acquired experience demonstrates that the MABEL dose should be especially considered, and receptor occupancy at this dose calculated, when the drug under development is a biological super-agonist molecule and when its mechanism of action suggests it can lead to uncontrolled enzymatic or cytokine cascade reactions.

2. In 2016, a Phase 1 trial of BIA 10-2474, a fatty acid amide hydrolase inhibitor, led to one death and caused serious neurological damage in few other healthy volunteers (Singh 2018).
   As a consequence, in 2017 the EMA updated the 2007 clinical guideline to further assist stakeholders in the transition from nonclinical to early clinical development and to identify factors influencing risk for new investigational medicinal products (EMEA/CHMP/SWP/28367/07 Rev. 1, 2017). This new version focused on the maximum exposure of healthy volunteers that should be within the estimated human pharmacodynamics dose range. This guideline, applicable to all new chemicals and biologics, somehow supersedes in the EU the previous US 2005 Guidance for Industry "Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers." The new 2017 version of the European clinical guideline mentions the PAD (Pharmacologically Active Dose) and/or ATD (Anticipated Therapeutic Dose) range that must be estimated in humans. In addition, the calculation of the MABEL, PAD, and/or ATD should consider target binding and receptor occupancy studies in vitro in target cells from human and the relevant animal species. Whenever possible, all relevant data should be integrated in a suitable modeling approach for the determination of the MABEL, PAD, and/or ATD.
   The bottom line is that the test program to be performed when developing such biologics should be adapted ("case by case") to the properties of the product in development and may be fundamentally different from the toxicological and more "conventional" or "classical" program designed to develop small molecules (see Table 2).

Comparison Between the Nonclinical Assessments of Biologics and New Chemical Entities

Table 2 compares the main nonclinical activities (pharmacology, pharmacokinetics, toxicology) to be performed with biologics and new chemical entities.

Pharmacology

Pharmacodynamics

For both NCEs and biologics, the intended pharmacological target is a main factor for deciding which test systems should be selected for the nonclinical development of the drug under investigation. The Sponsor should justify the relevance of the

Table 2 Nonclinical activities to be conducted for biologics vs. NCEs

Nonclinical activities (guidelines)	Biologics (ICH S6 applies)	New chemical entities
Pharmacology		
Primary pharmacodynamics (ICH M3(R2))	Yes, in vitro and in vivo, in at least a relevant species/model (case by case: humanized model if necessary)	Yes, in appropriate in vitro and in vivo models
Secondary Pharmacodynamics (ICH M3 (R2))	If relevant, in appropriate in vitro and in vivo models	If relevant, in appropriate in vitro and in vivo models
Safety pharmacology (ICH M3(R2), ICH 7A & B)	Yes (CNS, cardiovascular and respiratory systems, other systems if necessary)	Yes (CNS, cardiovascular and respiratory systems, other systems if necessary)
Pharmacokinetics and Toxicokinetics		
Analytical methods and Validation reports (EMEA/CHMP/EWP/192217/2009 Rev.1 Corr. 2)	Yes	Yes
Absorption (ICH S3A & B)	Yes	Yes
Distribution (ICH S3A & B)	Yes	Yes
Metabolism (ICH S3A & B) CDER guidance: safety testing of drug metabolites	No (degradations in small peptides and single amino acids)	Yes. If metabolite represents >10% of parent compound
Excretion (ICH S3A & B)	Yes	Yes
Toxicology		
Single-dose toxicity (ICH M3(R2))	Can be helpful to select the doses for repeated-dose toxicity	Can be helpful to select the doses for repeated-dose toxicity, however no need to reach LD_{50} levels anymore
Repeated-dose toxicity (ICH M3(R2), ICH S4A)	Yes (only in relevant species)	Yes (two species, rodent and non-rodent)
Genotoxicity (ICH S2(R1))	No	Yes (in vitro and in vivo)
Carcinogenicity (ICH S1A, B, and C)	Generally, not necessary	Yes (except for anticancer agents (ICH S9)
Reproductive and developmental toxicity (ICH S5(R2))	Yes, the program could be done in a single species	Yes (two species, rodent and rabbit)
Local tolerance (ICH M3 (R2), CPMP/SWP/2145/00)	Yes, stand-alone study usually not necessary	Yes, stand-alone study usually not necessary
Immunotoxicology (ICH S8)	ICH S8 does not apply, immunogenicity and anti-drug antibodies (ADA) assessments are needed	Yes, immunotoxicity assessment
Phototoxicity (ICH S10)	No	Yes, if light absorption, generation of UV reactive species, and skin/eye distribution is achieved

animal species to humans taking into account the target, its structural homology, its distribution, the signal transduction pathways, and the nature of pharmacological effects. The demonstrated pharmacodynamics (PD) characteristics of a drug under development in relevant animal model(s) will be considered as the nonclinical proof of concept for NCEs as well as biologics. In contrast to the key toxicological activities (including toxicokinetics evaluation), it is acknowledged that PD studies do not need to be Good Laboratory Practice (GLP)-compliant.

Safety Pharmacology

Safety pharmacology studies should be GLP-compliant (ICH S7A&B) and need to include assessment of effects on vital functions (cardiovascular system, central nervous system (CNS), and respiratory systems) to investigate undesirable effects of a substance and its metabolites on physiological functions based on exposure at low, medium, and high doses. For some products, the evaluation of safety pharmacology endpoints can be conducted as part of toxicology and/or pharmacodynamics studies. Cardiotoxicity is a major reason why NCEs fail to reach the market. In November 2018, the establishment of Q&As for the ICH E14 and ICH S7B guidelines was endorsed. ICH S7B and ICH E14 describe nonclinical and clinical risk assessment strategies concerning the pro-arrhythmic potential of non-antiarrhythmic test substances and contribute to the design of clinical investigations. These guidelines will inform on best practices for the design, conduct, analysis, interpretation and reporting of in vitro, in silico, and in vivo nonclinical assays (as the Comprehensive in vitro Pro-arrhythmia Assessment (CiPA) initiative), in order for these assays to influence nonclinical and clinical evaluations.

Pharmacokinetics and Toxicokinetics

Guidelines ICH S3A and B require a comprehensive knowledge of the absorption, distribution, metabolism, and excretion (ADME) in view of the interpretation of pharmacology and toxicology studies. Measurement of drug concentrations (PK determinations) in biological matrices is an important aspect of medicinal product development for both NCEs and biologics. Tissue distribution studies are essential, especially in relation to potential sites of action. For NCEs, the nonclinical characterization of human metabolites is only warranted when these metabolites are observed at exposures greater than 10% of total drug-related exposure at steady-state and at significantly greater levels in humans than the maximum exposure seen in the toxicity studies (FDA Guidance 2016, Rev 1, Safety Testing of Drug Metabolites). Such studies should be conducted to support phase 3 clinical trials. In contrast, the expected consequence of metabolism of biologics is the degradation to small peptides and individual amino acids. Therefore, the metabolic pathways are generally understood, and thus classical biotransformation studies as performed for pharmaceuticals are usually not needed.

In 2017, ICH issued a Q&A document to ICH S3A (Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies: Focus

on Microsampling). This document focuses on points to consider before incorporating the microsampling method in toxicokinetics (TK) studies and acknowledges its benefits and some limitations for assessment of TK in main study animals and its overall important contribution to the 3R benefits (Replacement, Reduction, and Refinement) by reducing or eliminating the need for TK satellite animals. This recommendation is now widely in place for any pivotal studies performed.

Toxicology

As a general rule, safety evaluation programs should only include the use of relevant species. Toxicity studies in non-relevant species may be misleading and are discouraged by the regulatory authorities. A relevant species is any animal model in which the test material is pharmacologically active, and thus knowledge of receptor/epitope distribution provides a general understanding of potential in vivo toxicity of biologics. A respective "case-by-case" cross-reactivity evaluation, in vitro and/or in vivo, by immunochemical, analytical, or functional tests between species and organs/ tissues/cells should be performed to help in the selection of the relevant test system. This would optimize the evaluation of toxicity arising from the binding to the receptor/epitope and any unintentional tissue cross-reactivity. An animal species which does not express the desired receptor/epitope may still be of some relevance for assessing toxicity if comparable unintentional tissue cross-reactivity to humans is demonstrated.

Repeated-Dose Toxicity

For NCEs, repeated-dose toxicity studies in two species are normally required. The studies should be designed to reflect the intended clinical use (duration and frequency of administration, clinical route of administration) and take into account the therapeutic indication. Frequency of administration is based on PD, PK, and toxicological profile. Dose levels often include a low (pharmacological), an intermediate, and a high (potentially toxic) dose. Of note, the regulation recommends multiple approaches for the highest dose of general, i.e., repeated-dose toxicity studies). When possible, this includes a maximum dose 50-fold the therapeutic target. A control group should also always be included.

For biologics, however, it is not rare to note that the pivotal toxicity program can be performed in a single species only (i.e., cynomolgus monkey). In case no relevant species can be determined, the use of transgenic animals expressing the human receptor or the use of homologous proteins should be considered. The information gained from the use of a transgenic animal model expressing the human receptor is optimized when the interaction of the product and the humanized receptor has similar physiological consequences to those expected in humans.

ICH M3(R2) requires treatment durations in non-rodents (9 months) and rodents (6 months) to enable long-term administration to humans (>6 months). It is noteworthy that for non-rodent animals, a treatment for 6 months may usually be

sufficient in the EU, yet to ensure a global approval, it is nevertheless preferable to treat non-rodents animals for 9 months.

For both NCEs and biologics, the evaluation of local tolerance (ICH M3(R2)) by the intended clinical route of administration is performed as part of the general toxicity studies. Stand-alone studies are generally not recommended.

Genotoxicity and Carcinogenicity

Genotoxicity tests can be defined as in vitro and in vivo tests designed to detect compounds that induce genetic damage by various mechanisms, such as gene mutations, chromosomal damage, or recombination. Extensive reviews have shown that many NCEs that are mutagenic in the bacterial reverse mutation (i.e., AMES) test are rodent carcinogens. To increase the sensitivity for detection of carcinogens, a battery approach has been implemented because no single test is capable of detecting all genotoxic mechanisms relevant in tumorigenesis. Thus, ICH S2(R1) states that if an assay for gene mutation is sufficient to support single-dose clinical trials with NCEs, a complete battery of genotoxicity tests, such as an AMES test, a cytogenetic test for chromosomal damage, and in vivo test for genotoxicity, should be completed before initiation of phase 2 trials, as well as to later on support the marketing of a product. Of note, genotoxicity studies are not considered essential to support clinical trials for therapeutics intended to treat patients with late stage or advanced cancer.

ICH S6(R1) states that biopharmaceuticals do not need to be tested for genotoxicity, as standard proteins and peptides are not supposed to induce damages at the DNA/chromosomal level.

Moreover, ICH M7(R1) has been introduced to define the maximal levels of daily mutagenic impurities which can be authorized within a NCE formulation. Importantly, genotoxicity studies are not applicable to biologics and therefore are presently not required (see ICH S6(R1)).

Conditions relevant for the carcinogenicity testing of NCEs are discussed in ICH S1A. In general, carcinogenicity studies should be conducted to support the marketing application and thus logically launched during phase 3. However, for pharmaceuticals developed to treat certain serious diseases, in order to speed up the development process, it is possible to discuss the timing with the agencies and conduct carcinogenicity studies post-approval. The basic scheme is one long-term rodent carcinogenicity study and one other study both supported with TK data (e.g., in vivo tests). A change to the current S1 guideline is foreseen and expected to introduce a more comprehensive and integrated approach to address the risk for human carcinogenicity. Ideally, this analytical approach will yield sufficiently instructive criteria for when a work-of-evidence option would be preferable to a 2-year bioassay in a development program, thereby improving assessment of human carcinogenic risk of pharmaceuticals and minimizing regulatory discordance across regions. As mentioned in ICH S6(R1), standard carcinogenicity bioassays are generally inappropriate for biologics. However, product-specific assessment of carcinogenic potential may still be needed depending upon the duration of clinical

dosing, patient population, and/or biological activity of the product (e.g., growth factors, immunosuppressive agents).

Reprotoxicity and Juvenile Animal Studies

As a consequence of the thalidomide disaster in the early 1960s (Vargesson 2015), reproduction toxicity studies should be conducted as appropriate for the population to be exposed (ICH S5(R2)). The goal is to reveal any adverse effect of the product on mammalian reproduction. The combination of studies (fertility, embryo-fetal, and peri-/postnatal assessment) selected should cover all stages of development from conception to sexual maturity. For NCEs, two species should be tested (rats and rabbits) to assess the potential of a NCE on the embryo/fetal development, as the thalidomide disaster revealed that mice are less sensitive to thalidomide than other species such as rabbits (Vargesson 2015). Observation should be performed from conception in one generation through conception in the following generation (complete life cycle).

For biologics, reproductive toxicity studies should also be conducted in accordance with the principles outlined in ICH S5(R2). However, one species only can be sufficient to address effects on embryo-fetal development. This guideline is currently under revision since 2015 (ICH S5(R3) EWG Revision of S5 Guideline on Detection of Toxicity to Reproduction for Human Pharmaceuticals). Interestingly, this draft version mentions the notion of Weight of Evidence (WoE). This is based on the fact that toxicity studies in pregnant animals are not always necessary for assessing the human risk of developmental toxicity of biopharmaceuticals. Therefore, the accumulated knowledge on target biology and molecule-specific pharmacokinetics should allow to accurately anticipate the effects of target activation by biopharmaceuticals using a WoE approach (Rocca et al. 2018). Such a WoE-based assessment should include all available data including target biology, pharmacokinetics, class effects, genetically modified animals, human mutations, and an exhaustive literature review. Such an evaluation may be sufficient to inform risk for specific clinical indications and patient populations, even though this approach is currently only applicable for oncology drugs and biologics. Noteworthy as well is that ICH S9 states to support the treatment of patients with late-stage or advanced cancer, generally neither warrants a fertility study nor any peri- and postnatal toxicology study. These are clear examples for modernizing testing paradigms to enhance human risk assessment while also potentially reducing animal use, notwithstanding that there are further areas where the guideline could be revised or amended for greater clarity, as well as to align more fully with other more recent ICH guidelines such as ICH M3(R2), ICH S6 (R1) as well as ICH S9.

As regards juvenile animal studies (JAS), a draft 2 guideline (ICH S11) was issued in 2018 to ultimately harmonize regional guidelines from various agencies to reach an agreement on the need for, timing of, and design of JAS and thus allow a common development program of medicines for the pediatric population. This is particularly relevant in the EU, where filing for a marketing authorization, even for an indication intended for adults only, will be systematically refused by the EMA in the absence of a Pediatric Investigation Plan (PIP) previously accepted by the Pediatric Committee of the agency.

Immunogenicity and Immunotoxicity

In contrast to the majority of NCEs, many biologics intended for human are immunogenic in animals. The immunogenicity of biologics can cause hypersensitivity responses, anaphylaxis, and infusion reactions (Rosenberg 2003). Anti-drug antibody (ADA) responses could affect the efficacy and/or safety of protein therapeutics and/or complicate interpretation of the toxicity, pharmacokinetic, and pharmacodynamic data. It is also known that particular glycosylation patterns might be immunogenic and some protein aggregates might trigger immunogenicity. Animal models are increasingly used to study immunogenicity of therapeutic proteins. They are employed as predictive tools to assess immunogenicity during drug development and have become vital in studying the mechanisms underlying immunogenicity of therapeutic proteins. However, the use of animal models needs critical evaluation (Brinks 2011). Because of species differences, the predictive value of these animal models is limited.

It is widely acknowledged that biologics often reveal their real immunotoxicity potential for humans only during clinical studies. The predictive value of animal studies and traditional in vitro screens is thus questionable. Despite these limitations, antibody levels associated with administration of biologics should be measured during repeated-dose toxicity studies. Antibody responses should be characterized (titer, number of responding animals, neutralizing or non-neutralizing), and their appearance should be correlated with any pharmacological and/or toxicological changes. Specifically, the effects of antibody formation on PK/PD parameters, incidence and/or severity of adverse effects, complement activation, or the emergence of new toxic effects should be considered when interpreting the data. Possible pathological changes related to immune complex formation and deposition should be evaluated.

In line with the above comments, both the FDA and the EMA recently issued guidelines on these topics. The FDA released a guidance on the development of biologics and biosimilars (FDA Guidance for Industry 2019). The FDA recommends a multi-tiered testing approach, and the document spells out the development and validation of screening, confirmatory, titration, as well as neutralization assays. In the EU, the guideline on the immunogenicity assessment of therapeutic proteins (EMEA/CHMP/BMWP/14327/2006 Rev 1) states clearly that the current predictive value of animal studies for evaluation of immunogenicity of a biological medicinal product in humans is low due to differences between human and animal immune systems and to immunogenicity of human proteins in animals. The development of adequate screening and confirmatory ADA assays to measure immune responses against a therapeutic protein is the basis of the evaluation of immunogenicity.

As regards NCEs, dedicated immunotoxicity studies are mandatory only when a cause for concern is identified in the repeated-dose toxicity studies (ICH S8). In this case, additional immunotoxicity studies should be performed to verify the immunotoxic potential of the compound, completed before exposure of a large population (before phase 3).

The Standard for Exchange of Nonclinical Data (SEND) Format

As regards the submission of toxicology and safety reports to regulatory agencies, the SEND initiative has been recently implemented by the FDA (Demollari 2019; Carfagna et al. 2020) in order to submit nonclinical data in a structured manner. The format was created by the Clinical Data Interchange Standards Consortium (CDISC). The governing document of the SEND standard is the Standard for Exchange of Nonclinical Data Implementation Guide (SENDIG). It describes the rules for providing standardized data according to the study data tabulation model (SDTM) for nonclinical studies.

The FDA requests that all nonclinical data from safety studies started on or after December 17, 2016, should be presented according to SEND. This applies to New Drug Applications (NDAs), Biologic License Applications (BLAs), and Abbreviated New Drug Applications (ANDAs), and for Investigational New Drugs (INDs) after December 17, 2017. Our strong advice to Sponsors is to ensure that all single/repeated-dose toxicity, carcinogenicity, and safety pharmacology (on cardiovascular and respiratory evaluation) data are provided in adequate format. The modalities should be discussed and aligned in-house or with the selected Contract Research Organization where the study will be performed.

Impact of Manufacturing and Formulation Changes on the Development Process

The performance of safety bridging strategies within batches of the same biological produced at different scales is a key element to master in order to obtain clinical trial and marketing authorization. The use of cells of human, animal, or even plant origin for the production of biologics is subject to potential contamination. A change in manufacturing process and/or of formulation of the product represents a potential risk for patients (such as immune-suppression, immuno-stimulation, hypersensitivity, and autoimmunity). Particular attention must be paid to the characterization, purity, and stability of the starting materials, as well as the presence of aggregates. Products should be tested for viral safety (ICH Q5A(R1) Quality of Biotechnological Products: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin) and genetic stability (ICH Q5B Quality of Biotechnological Products: Analysis of the Expression Construct in Cell Lines Used for Production of r-DNA Derived Protein Products). A European guideline on the requirements for quality documentation concerning biological investigational medicinal products in clinical trials was adopted in 2008 and revised in 2018 (EMA/CHMP/BWP/534898/2008 rev.1 corrigendum).

The production process must provide relatively large amounts of test material. The degree of comparability of the test material from batch to batch in the development program requires an early validation of the production and testing methods as well as the precise definition of the product specifications (EMEA/ CHMP/BMWP/101695/2006). An early well-designed bridging strategy in terms of upscale process is preferable to a subsequent full test program.

Alternative Methods Including In Silico Evaluation

Animal models are widely used for a long time for toxicity testing. However, in vivo animal tests are constrained by time, ethical considerations, and financial burden. ICH M3(R2) states that "consideration should be given to use of new in vitro alternative methods for safety evaluation. These methods, if validated and accepted by all ICH regulatory authorities, can be used to replace current standard methods." Therefore, not surprisingly, this last decade has seen the emergence of a paradigm shift: in line with the 3Rs principle (reduce/refine/replace), regulatory agencies now appear more open to alternative approaches to decrease the number of animals during the nonclinical development of NCEs and biologicals. This change is particularly apparent in the draft guidance ICH S5(R3), which once adopted may take into consideration data from, e.g., qualified alternative in vitro and non-mammalian assays, in combination with one or more in vivo mammalian embryo-fetal development studies. For tolerance assessment, alternative methods on 3D reconstructed human epithelium (ocular or cutaneous) can already replace the previous eye and skin irritation studies in rabbit.

In silico toxicology is one type of toxicity assessment that uses computational methods to analyze, simulate, visualize, or predict the toxicity of chemicals. In silico toxicology aims to complement existing toxicity tests to predict toxicity, prioritize chemicals, guide toxicity tests, and minimize late-stage failures in drug design (Raies and Bajic 2016). In silico toxicology methods involve a wide variety of computational tools: databases for storing data about chemicals, their toxicity, and chemical properties; software for generating molecular descriptors; simulation tools for systems biology and molecular dynamics; modeling methods for toxicity prediction; modeling tools such as statistical packages and software for generating prediction models; expert systems that include pre-built models in web servers or standalone applications for predicting toxicity; and visualization tools. A plethora of databases (almost 1000) exist online for in silico drug safety assessment. A recent review (Pawar et al. 2019) provides a comprehensive listing of the key in silico data resources such as chemical identity and properties, mechanism of action, toxicology, exposure, ADME properties, clinical trials, pharmacovigilance, patent-related databases, protein-protein interactions, and, finally, databases related to animal alternatives in support of 3Rs policies.

Finally, based on our own experience, the FDA seems more advanced than the EMA as regards modeling and simulation (M&S), having already identified M&S tools as one of the priorities to improve in the FDA's 2011 Strategic Plan for Advancing Regulatory Science. This plan included the need for developing also clinical trial simulation models that can reveal interactions between drug or device effects, patient characteristics, and disease variables influencing outcomes, as well as development of data management tools to inform computer model development, clinical risk prediction, and regulatory decision-making (Rousseau et al. 2019).

Environmental Risk Assessment (ERA)

An Environmental Risk Assessment is required by EMA since 2006 for all new marketing authorization applications (MAA) for a medicinal product through a centralized, mutual recognition and decentralized and national procedure regardless of its legal basis. This affects all new products (with some exceptions like vitamins, amino acids, peptides, electrolytes, and herbal products), including already marketed drugs, if, e.g., a new indication results in significant increase in their extent of use (EMEA/CHMP/SWP/4447/00 corr 2, 2006). A revised draft guideline is in preparation with a consultation period closed in June 2019 (EMEA/CHMP/SWP/4447/00 Rev 1, 2018). These guidelines follow a risk-based approach based on environmental release of the pharmaceutical, with testing dictated by partitioning; solubility; persistent, bioaccumulative, and toxic (PBT) characteristics; and endocrine activity.

Even if the ERA requirements in the USA may appear less stringent than in the EU, the FDA stipulates also that a risk assessment or categorical exclusion claim should accompany every IND, NDA, or BLA (FDA/CDER/CBER/1998). In 2016, FDA supplemented this guidance (FDA/CDER/2016) by addressing specific considerations for drugs that have potential estrogenic, androgenic, or thyroid hormone pathway activity in the environment.

Conclusion and Recommendations

Biologicals can provide more innovative, effective, and targeted therapies for numerous diseases than NCEs. In order to detect any potential toxicity of these promising products, the determination of the safe dosage at the start of clinical studies and the establishment of dose–response relationships, a rationale "case-by-case" nonclinical testing strategy, should be put in place taking into account not only ICH S6(R1) (Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals) but also all other guidelines listed in this chapter. Biologicals differ in many aspects from the more conventional NCE drugs, because of their species- and tissue-specific characteristics and their immunogenicity potential due to their particular nature and complex mode of production.

With biologicals, the required safety testing may appear more science-driven and designed around the product and the predicted safety issues resulting from the biology (mechanism of action), rather than being a standard list of tests. This is because toxicity with biologicals is, in the vast majority of cases, secondary to the pharmacology, whereas NCEs can have off-target effects unrelated to the biology or pharmacology.

It is also our view that, in the next decade(s), the importance of alternative methods (in vitro and in silico) to assess the toxicity of our future medicines will considerably expand. Such a (r)evolution is already initiated and may not only impact animal research. Indeed, the FDA initiated a pilot program known as the model-informed drug development (MIDD) in which the agency meets with drug developers to discuss and agree on which key program decisions can be supported

by mathematical models and simulations. The FDA may even accept in the near future some selected clinical trials performed with virtual patients.

Finally, in order to avoid critical issues at the time of marketing authorization application, we strongly advise any drug developer to request a timely scientific advice meeting with a regulatory agency to discuss and find an agreement on the relevance of the nonclinical development program they intend to perform.

Cross-References

► Microbiome Product Toxicology: Regulatory View on Translational Challenges
► Toxicological Aspects in the Regulation of Gene Therapy Medicinal Products

Disclaimer Any views and opinions expressed in this chapter are those of the authors and do not necessarily reflect those of any institutions the authors are associated with.

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Sensitive Humans Versus Average Persons in Toxicology

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Alexander Eckhardt

Contents

Abstract

The sensitivity of human beings to toxic insults or pharmaceuticals varies considerably. This fact has a series of reasons, e.g., health status, age, body weight, as well as genetic background. An above average sensitivity against a noxious substance can have severe or even lethal consequences. Sensitivity varies not only between individuals but also between populations as a whole. Therefore, it is compulsory for toxicologists to take these differences into account when establishing limits for toxic compounds.

A. Eckhardt (✉)
Toxicology of Drinking Water and Swimming Pool Water, German Environment Agency, Bad Elster, Germany
e-mail: Alexander.Eckhardt@uba.de

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Introduction

The genetic background of human beings from all over the globe varies quite a lot. In addition, different environmental conditions and cultural habits can influence the susceptibility against a substance either of natural origin or manmade. On the first glimpse, these differences are not a problem, but from a toxicological and/or pharmacological point of view matters are different. Besides accidents and (attempted) suicides, there is usually no toxicological data based on human exposure to high doses of xenobiotics. We thus have to rely on animal testing. A group of animals used for testing is usually well defined, regarding genetic background, age, and body weight. The problems which may arise when the data acquired are transferred to humans are based on the fact that humans are considered a good deal more genetically diverse than animals which are used for toxicity testing. Defining an "average person" is already impossible in a small population, let alone on a worldwide scale. Therefore, safety margins big enough to include those rare people with a very high sensitivity against a certain compound have to be set.

This chapter deals with the different reasons for diverging susceptibility in humans. Probably the most important cause is genetic polymorphisms, followed by parameters like age, health status, and body weight. Toxicological implications are discussed, followed by a special focus on children.

Genetic Polymorphism

The effects of genetic polymorphisms vary greatly depending on the enzyme concerned. It is common knowledge that many adult Asians (~80%) have developed lactose intolerance. This might be undesirable for the respective person, but it's usually not life threatening. Things are getting more complicated when we look at cytochromes for example. This big class of enzymes encompasses at least 18 families (http://drnelson.uthsc.edu/human.P450.table.html), which in turn can be divided into two major groups: cytochromes that are responsible for synthesizing endogenous substances like steroids and fatty acids as well as enzymes dealing with xenobiotics (Lin and Lu 2001). Among those xenobiotics are toxicants like aryl hydrocarbons from tobacco smoke as well as pharmaceuticals. Pharmaceuticals are mainly metabolized by three cytochrome families: CYP1, CYP2, and CYP3, with the latter two accounting for about 50% of a person's hepatic cytochrome make-up. Only two of almost 60 known isoforms seem to be well preserved: CYP1A1 and CYP2E1. The reason might be that the former is part of the cell cycle, whereas the latter is part of gluconeogenesis.

Similar variability is seen in other important molecule classes, from glutathione S-transferases (GSTs), to CC chemokine receptor (CCR) and nicotinic acetylcholine receptors (Dasari et al. 2018; Qidwai 2016; Wei et al. 2015).

Genetic variations can account for differences in drug uptake, metabolism, and drug-receptor interaction. Each of these differences can lead to adverse drug effects. The estimate that more than 10% of the admissions to internal medicine departments

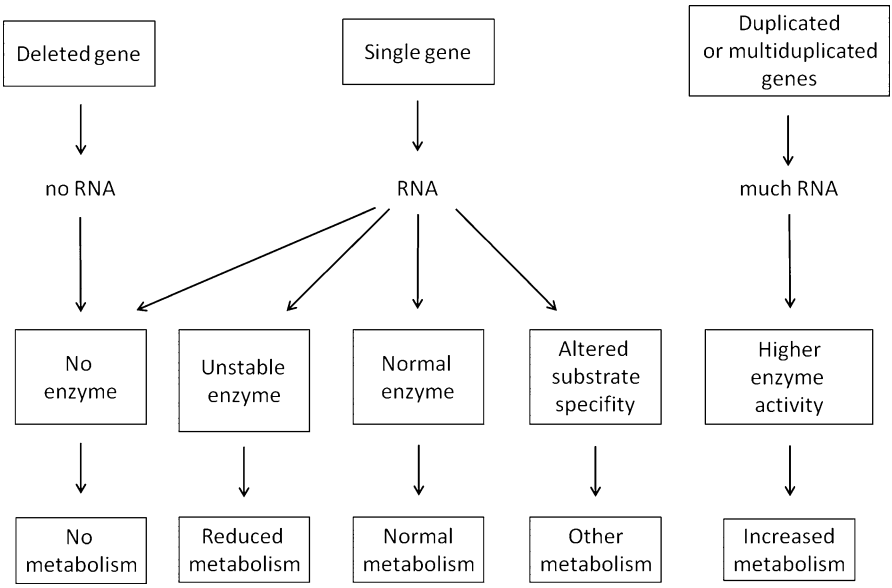

Fig. 1 Possible causes for differences in cytochrome P450 activity in different subjects, (Ingelman-Sundberg 2001, redrawn)

in Swedish hospitals are due to adverse drug effects, gives an idea about the importance of the knowledge of polymorphisms (Mjörndal et al. 1999). Even more alarming is the US assessment that about 100,000 deaths annually are caused by adverse drug effects (Ingelman-Sundberg 2001). An overview of possible effects of gene mutations in cytochromes can be seen in Fig. 1.

One prominent example for genetic polymorphisms is CYP2D6, which metabolizes a variety of pharmaceuticals like anti-depressants, anti-hypertensive drugs, and anti-arrhythmic agents. Altogether it is estimated that CYP2D6 metabolizes about 25% of all pharmaceuticals on the market (Byeon et al. 2018). The field of CYP2D6 is rapidly expanding. When Byeon et al. wrote their paper, 113 allelic variants of this cytochrome were known (Byeon et al. 2018). The linked webpage (http://www.pharmvar.org/gene/CYP2D6), in June 2020, indicated almost 140 allelic variants. Of these, nine alleles are most important and they show different levels of activity: *1 and *2 are fully functional alleles, *3, *4, *5, and *6 are nonfunctional alleles and *10, *17, and *41 are reduced functional alleles (Byeon et al. 2018). Variant CYP2D6*17 has a frequency of about one third in people of African origin whereas it is almost nonexistent in Caucasians (Ingelman-Sundberg 2001). Since this variation results in reduced substrate affinity, severe adverse drug effects have to be considered before prescription. One of the pharmaceuticals affected is the neuroleptic perphenazine, with variations of about tenfold in patient's serum, when comparing standard to poor metabolizers. CYP2D6 is also known for being a cytochrome encoded by multiple, i.e., up to 13, gene copies. Consequently, the individual concerned can be a very rapid metabolizer, which would result in only limited

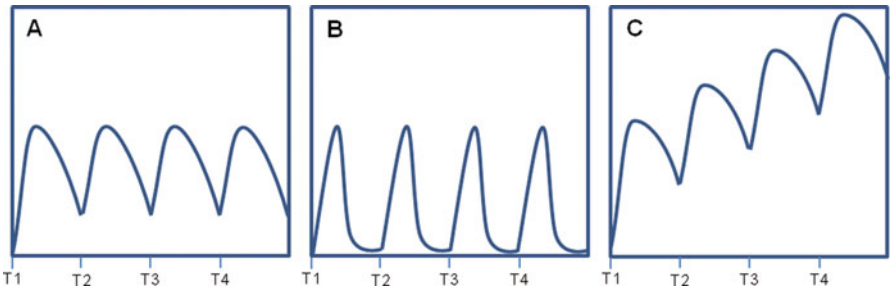

Fig. 2 Effects of different metabolic rates on the plasma level of a compound: normal metabolizer (**a**), rapid metabolizer (**b**), or slow metabolizer (**c**)

effects of conventional drug dosing regimens. Different plasma concentrations of a drug given at four intervals as expected by standard-, rapid-, or slow metabolizers are shown in Fig. 2. The differences in CYP-regulated metabolism between two individuals can amount up to 100-fold for a single drug. A meta-analysis on global distribution of CYP450 alleles can be found in Zhou et al. (2017).

There are also polymorphisms of the steroidogenic cytochromes. But due to their severely debilitating or even fatal effects, they are generally regarded as genetic defects (Guengerich 2002).

Additional Parameters

Variations in the plasma level of the same pharmaceutical of up to 1000-fold between two persons with identical body weight, have not only genetic reasons (Ingelman-Sundberg 2001). In addition, age, (patho) physiological, nutritional, and environmental effects have to be taken into account. One class of xenobiotics that is known to increase cytochrome activity is polyaromatic hydrocarbons (PAHs). The induction observed can have different reasons, depending on the source of the PAHs, which can be rather divers: it could be the 8 oz. steak from the barbeque, the cigarette or the chemical plant in the neighborhood. All this results in an uptake of PAHs, which in turn induce CYP1A activity. Contrary to lab animals used in pharmaceutical testing, the diet of humans is very diverse, as are possible effects induced by food. Therefore, finding the reason for variations in two persons is very complicated and sometimes a virtually impossible task.

Toxicological Implications

The main task of CYPs in dealing with xenobiotics is the detoxification of these compounds. Thereby hydrophilic groups such as hydroxyl-groups are added to the original substance to increase its water solubility. Thus, renal clearance can be facilitated. Alas, this strategy has a drawback: adding one or more hydroxyl-groups

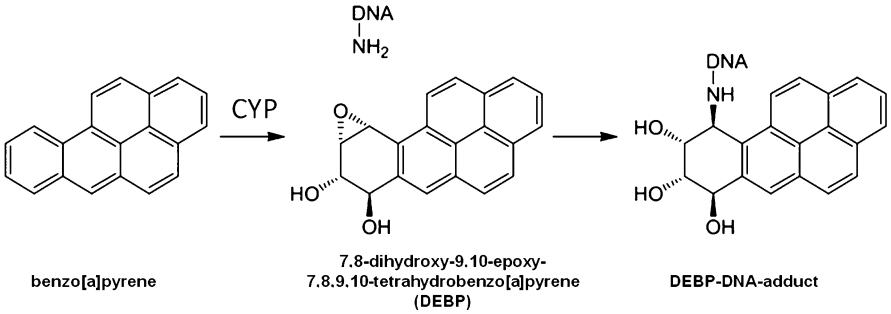

Fig. 3 Activation of benzo[a]pyrene by adding 4 hydroxyl-groups and formation of DNA adduct (http://www.chemgapedia.de/vsengine/media/width/713/height/190/vsc/de/ch/4/cm/funktgruppen/bilder/benzpyren_dna.svg.jpg)

to a substance can also result in an increased reactivity. Due to its lipophilic properties, benzo[a]pyrene has to be subjected to several activation steps by CYPs. Since benzo[a]pyrene can be metabolized by several cytochromes as well as epoxide hydrolase, several different metabolites are formed. One of the metabolites formed is 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (Fig. 3). The epoxy-group is very reactive and can easily bind to amino groups of proteins or, more dangerous, DNA. Although the epoxide can be eliminated via glutathione transferases (GSTs), increased amounts of DNA- adducts can be formed, e.g., when sufficient amounts of GSTs are not available.

Depending on the activity of the enzymes involved in this reaction, the amount of DNA adducts formed can vary widely. With more adducts formed, the risks of unrepaired or inaccurately repaired DNA damage increases. Once the damage is done, there are three alternatives on the cellular level: apoptosis, correct repair, or inaccurate repair, resulting in mutation and possibly cancer (Fig. 4). Although deadly for a single cell, apoptosis is very useful for the organism as a whole, since the damage will not be inherited to daughter cells, thereby eliminating the risk of cancer.

Polymorphisms in cytochromes can result in severe consequences, when pharmaceuticals are not metabolized as expected by the physician. This can result in very different plasma levels as shown in Fig. 2. Antibiotics, like erythromycin, are metabolized by CYP3A4. In a worst-case scenario, too much CYP3A4 can lead to septic shock, because the plasma level of the antibiotic is insufficient. A second problem arising from rapid metabolism can be an indirect effect. Acetaminophen also called paracetamol, for example is metabolized by CYP2E1. It is known that the inter-individual concentration of CYP2E1 can vary by a factor of 12. High levels of CYP2E1 can lead to an accumulation of N-acetylbenzoquinoneimine, a major product in the metabolism of paracetamol. This can result in irreversible hepatic necrosis, when the liver cannot provide enough glutathione for the detoxicating conjugation of *N*-acetylbenzoquinoneimine. Table 1 gives an overview of cytochromes, their variability, substrates, inducers, and inhibitors.

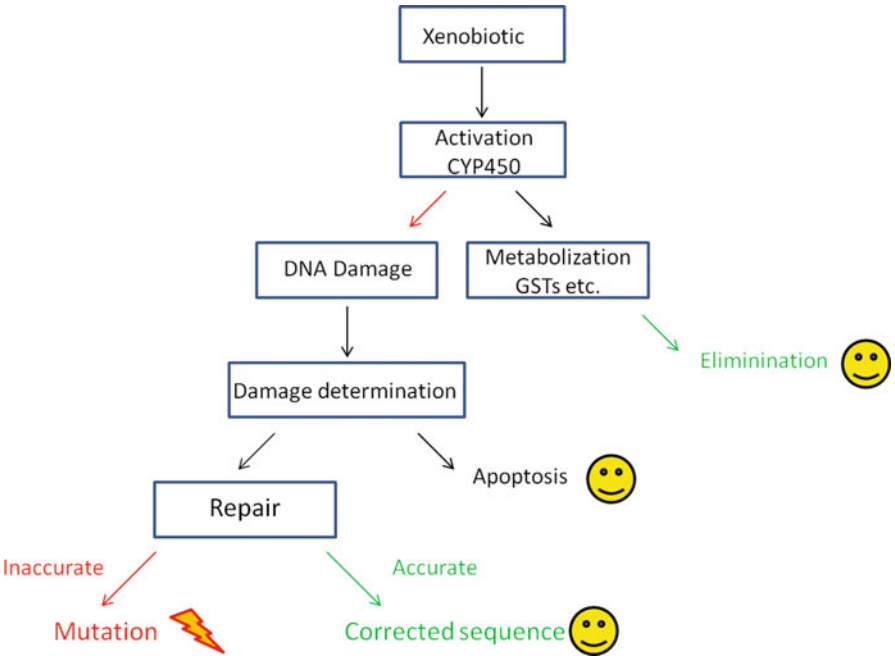

Fig. 4 Possible pathway after activation of a xenobiotic by CYP

Table 1 Inter individual variability of cytochrome activity. (Data from Lin and Lu 2001; Pelkonen et al. 2008)

P450-enzyme	Variability	Substrate	Inhibitor	Inducer
CYP1A2	20-fold	caffeine	ciprofloxazine	smoking
CYP2A6	>50-fold	nicotine	pilocarpine	phenobarbital
CYP2B6	>50-fold	nicotine	17-α-ethinyl estradiol	phenobarbital
CYP2D6	>50-fold	codein	fluoxetine	?
CYP2E1	12-fold	ethanol, acetaminophen	pyridines	ethanol(!)
CYP3A4	8-fold	testosterone	grapefruit juice	phenobarbital

Since cytochromes are in contrast to many other enzymes inducible, the interplay between enzyme and its substrate(s) becomes even more complex. Inhibition or induction can be triggered by pharmaceuticals like fluoxetine, phenobarbital or 17-α-ethinyl estradiol. But not only medications can cause additional variations in CYP activity but also dietary products are known to influence cytochrome activity. A very effective inhibitor of several cytochromes is grapefruit juice. The inhibition of CYP3A4, CYP1A2, CYP2C9 and CYP2D6 was observed after treating liver microsomes with grapefruit juice. Due to the fact that at least four different CYPs are

inhibited by the juice, a broad spectrum of pharmaceuticals is affected: Calcium channel blockers, immunosuppressive drugs, sedatives and others. Cytochrome inhibition can increase the bioavailability of these pharmaceuticals up to fivefold, e.g., for the calcium antagonist felodipine (Bailey et al. 1991). Although oranges are closely related to grapefruits, these effects are not seen after consumption of orange juice (Tassaneeyakul et al. 2000). Grapefruit ingredients like naringin cause the interactions.

Susceptibility in Children

Physiological Differences

It is well justified to assume that there are biological reasons why children, and especially newborns, can be more sensitive towards a comparable toxicological stress than adults. Compared to adults, their consumption of food, water and oxygen is increased in relation to their body weight. Moreover, children are on average physically much more active. Consequently, their exposure to environmental stress is also elevated. What makes matter worse is the fact that especially in the first 6 months the ability to metabolize xenobiotics may not be fully developed.

Increased Susceptibility in Fetuses, Babies, and Children

It is common knowledge that exposure during pregnancy to certain substances results in damage selectively in children. Examples are smoking, alcohol consumption, or uptake of pharmaceuticals which lead to neurotoxic or teratogenic effects or developmental retardation.

Less is known about intoxication of children with chemicals, like pesticides or food additives that are used only in restricted applications. Children are more susceptible against high acute doses of the pesticide chlorpyrifos, but on the other hand, less or as sensitive as adults against repeated low dose exposure. Although many persistent organic compounds have been banned, and their prevalence is reduced, neurotoxins, like methylmercury, that damage the developing brain of children, are still on the agenda in regulation. Lead is of special concern in children for two reasons. Firstly, the adsorption in a child's gastrointestinal tract is higher than in an adult and, secondly, the central nervous system of children is four times more susceptible than that of an adult (ATSDR 2019). Therefore, the main focus is on neurotoxic effects in children, when it comes to discussing tolerable lead concentrations. Consequently, the new EU drinking water directive to be published in 2020 will lower the maximum allowable lead concentration to 5 µg/L instead of 10 µg/L and therefore be stricter than WHO Guidelines. The rationale behind this is the developmental toxicity of lead putting fetuses and children at particular risk.

There is only limited evidence from literature, that low doses of easily metabolized and excreted substances are more toxic to children than to adults. Damage

through alcohol or cyanoses induced by nitrate/nitrite from private wells are known examples.

According to current knowledge about ontogenic development of human metabolism, children up to the age of 6 months are generally more susceptible against toxic insults than adults. This is caused by the fact that biotransformation and elimination is slower, which in turn results in higher plasma levels of many chemicals and pharmaceuticals. An example for this is an enzyme called NADH cyb5r reductase which converts nonfunctional methemoglobin to functional hemoglobin. Infants under the age of 6 months have much lower level of this enzyme than adults, rendering them susceptible to the toxic effects of nitrate/nitrite (Fossen Johnson 2019). In addition, fetal hemoglobin is more rapidly converted to methemoglobin than its adult counterpart. Therefore, infants have a double disadvantage when they have to deal with nitrate uptake.

The metabolic capacity for dealing with many xenobiotics is already established prior to or at the time of birth, but the capacity is smaller and the enzyme patters can be different. At the age of 6 months, the metabolism of children is developed well enough, that there are usually no important variations in the toxic susceptibility compared to adults. Nevertheless, the susceptibility of organs like brain, bones and hormonal system can remain different until sexual maturity. Children can also be less susceptible to chemicals or pharmaceuticals, when certain receptors or final metabolic capacities are not yet fully developed.

Due to age specific behavior, oral exposure to chemicals is increased in children. Since especially small children are trying to put almost everything, they can grab a hold on into their mouth, households, and public playgrounds have to be taken into account as additional sources of exposure. The risk is dependent on a child's susceptibility and the exposure conditions. Provided that the chemical exposure remains below the threshold above which detoxification mechanisms of the organism are overstrained and toxic effects are triggered, the hazard is the same as for an adult. During the first half-year, when the human organism is generally most vulnerable, the exposure to chemicals is, also because of limited mobility, rather low. Therefore, exposure to environmental risks can be considered lower the in older children.

Regulatory Considerations

Children of all ages are still in the process of maturation. This has to be taken into account, when assessing the risk of substances that impair the development of organs when children could be exposed against them. Based on this, the US-EPA considers the implementation of an extra safety factor of 10, when the data on a compound does provide reliable information about a toxicological threshold in children.

Moreover, there are additional regulations for the special protection of children, e.g., in regulations concerning food and toys (Diätverordnung, Spielzeugverordnung in Germany). In accordance with the WHO "Guidelines for Drinking-Water Quality" (WHO 2017), some parameters of the German drinking water directive

(Trinkwasserverordnung), like the concentration of nitrate and copper, were set to meet the requirements of children.

Cross-References

▶ Importance of Xenobiotic Metabolism: Mechanistic Considerations Relevant for Regulation

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Default Factors in Quantitative Risk Assessment

41

Klaus Schneider and Leonard Ritter

Contents

Abstract

Only rarely is sufficient toxicological knowledge available on a risk of interest. In cases where toxicological data are incomplete for a specific quantitative risk assessment, the assessment may also draw on general scientific knowledge gained from experience with other chemical substances. However, this approach of extrapolation, using default factors based on empirical evidence, is not without controversy.

Keywords

Risk assessment · Assessment factors · Duration extrapolation · Interspecies extrapolation · Intraspecies extrapolation

K. Schneider (✉)
Forschungs- und Beratungsinstitut Gefahrstoffe GmbH, Freiburg, Germany
e-mail: klaus.schneider@fobig.de

L. Ritter
School of Environmental Sciences, University of Guelph, Guelph, ON, Canada
e-mail: lritter@uoguelph.ca

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Introduction

Suitable human data for the relevant risk group is frequently absent when performing quantitative risk assessment. Risk assessment is therefore often based on test animal data which need to be evaluated with regard to the risk group in question. Such an assessment and estimation was first performed in the USA as early as 1954 based on defined principles. Also since the 1950s, bodies of the World Health Organization (WHO), the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and the Joint FAO/WHO Meeting on Pesticide Reviews (JMPR) have set ADI values (▶ Chaps. 44, "Health-Based Threshold Values Versus MOS in Toxicology," and ▶ 99, "Limit Values and Guideline Values in Regulatory Toxicology") for food additives and pesticide residues in food as at least 1/100 of the NOAEL (▶ Chap. 22, "Dose-Response Analysis: Identification of Threshold Levels for Chemicals") in experimental animals.

This factor-based assessment with the help of conventions has been the subject of discussions again and again since then. On the one hand, it is rejected as unscientific, while on the other hand attempts have been made at the same time to justify the factor physiologically or empirically. For this purpose, the overall factor was subdivided into individual factors. For example, the WHO typically defines, in purely formal terms, the traditional intraspecies factor of 10 as a quantity made up of 3.2 for toxicokinetic variability times 3.2 for toxicodynamic variability (= 10), without further justifying the size of the factor. The WHO applied a similar approach in the case of the interspecies factor (4.0 for toxicokinetics times 2.5 for toxicodynamics, see Table 3).

Depending on the regulatory context (e.g., occupational safety and health or public health), different factors must be chosen with regard to the relevant population at risk which the risk assessment proposes to protect (healthy workers or the general population). However, it is generally acknowledged that substance-specific information should be used to replace default factors whenever possible (Bhat et al. 2017). WHO developed guidance on how to derive so-called chemical-specific adjustment factors (WHO 2005).

The following focuses on public health aspects. But also for deriving occupational exposure levels (OELs), empirically derived factors are increasingly used (Maier et al. 2015; Schenk and Johanson, 2018; ECHA 2019).

Nomenclature

In general, a distinction can be drawn between two types of assessment steps: physiologically/empirically based assessment steps and assessment steps that cannot be scientifically or empirically validated (Kalberlah and Schneider 1998):

1. Physiologically/empirically based assessments use toxicological data and extrapolations to an expected level on the basis of those data (e.g., lowering of the

Table 1 Subdivisions of extrapolations and qualitative aspects in quantitative risk assessment

Physiologically/empirically based extrapolations	Qualitative aspects
Duration extrapolation	Data quality/completeness of database
LOAEL/NAEL extrapolation	Type/severity of effects
Interspecies extrapolation	Grounds for suspicion
Intraspecies extrapolation	

Table 2 Default factors and underlying assumptions for equipotent extrapolation (scaling) of data from animal experiments to humans

	Assumed body weight of experimental animal[a]		Allometric scaling factors (caloric demand scaling)	
	US EPA[b]	ECHA[c]	US EPA	ECHA
Mouse/human	30 g	30 g	7	7
Rat/human	250 g	250 g	4	4
Hamster	–	110 g	–	5
Guinea pig	500 g	800 g	3	3
Rabbit/human	2.5 kg	3.8 kg	2	2.1
Monkey/human	–	4 kg	–	2
Dog/human	–	18 kg	–	1,4

[a]Body weight assumed for humans: 70 kg
[b]US Environmental Protection Agency: EPA/630/P-02/002F, December 2002, Final Report
[c]European Chemicals Agency: ECHA Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.8, Version: 2, 2010

NOAEL to extend the test period to lifelong exposure). This quantitative assessment should include a comprehensive interpretation of empirical data.

2. Additional, qualitative aspects, not based on the same data, are considered in order to safeguard against uncertainties (e.g., consideration of an incomplete data base to safeguard against potential, untested effects).

Table 1 provides an overview of how extrapolations and qualitative aspects are subdivided.

According to its "Guidance document for use of data in dose/concentration-response assessment," the WHO distinguishes between the terms "adjustment factor" for chemical-specific factors and "uncertainty factor" for default factors. The WHO applies these factors to account for both uncertainties and variabilities. The US Environmental Protection Agency (US EPA) also uses the term "uncertainty factors" for its assessments but also applies "empirically derived scaling factors" as addressed in Table 2. A possible reason for replacing the previously common term "safety factor" with "uncertainty factor" may have been to avoid conveying the (wrong) impression of absolute safety. The European Chemicals Agency (ECHA) uses the term "assessment factor" for factor-based "extrapolations."

Extrapolations

The following extrapolations may have to be performed in quantitative assessment:

- Duration extrapolation (e.g., from subchronic to chronic exposure)
- Extrapolation from an available LOAEL (lowest observed adverse effect level) to a NAEL (no adverse effect level) as the desired level of protection (▶ Chap. 22, "Dose-Response Analysis: Identification of Threshold Levels for Chemicals")
- Interspecies extrapolation (from experimental animal to human)
- Intraspecies extrapolation (from groups with average susceptibility to groups with increased susceptibility)

Such extrapolations are a component of most assessment concepts. Standard or default values of up to 10 are usually invoked for these extrapolation steps (see Table 3), which are described in more detail below; these default values should, however, be adjusted to reflect substance-specific knowledge as far as possible. The following rationales for applying these assessment steps, and the following empirically derived quantifications, based on the analysis of relevant data on a large number of substances, have been under discussion:

Table 3 Comparison of default factors used by different organizations in quantitative risk assessment (systemic effects)

Factor for	US EPA[a]	WHO[b]	ECHA[c]
LOAEL-NAEL	10 alternatively BMD[d]	Up to 10 alternatively BMD	Preferred BMD
Intraspecies-variance	10, reduced to 3 if based on data from susceptible subgroups	Up to 10 (3.16×3.16 for toxicokinetics and toxicodynamics)	10
Interspecies-variance	10 toxicodynamic component $10^{0.5}$ (≈ 3)	Up to 10 (4.0×2.5 for toxico-kinetics and toxicodynamics)	Factor for allometric scaling (Table 2) Remaining diff. 2.5
Subchronic to chronic	10	1–100	2 (geometric mean)
Additional safety	Modifying factor > 0–10		1
Combination	Multiplicative[e] (RfC, max. 3000; RfD, max. 10,000)	Multiplicative	Multiplicative

[a]US Environmental Protection Agency: EPA/630/P-02/002F, December 2002, Final Report
[b]World Health Organization: Environmental health criteria 170 (IPCS) 1994
[c]European Chemicals Agency: ECHA Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.8, Version: 2, 2010
[d]*BMD* benchmark dose
[e]*RfC* reference concentration, *RfD* reference dose

Duration Extrapolation

Duration extrapolation will be necessary if, in a risk assessment designed to cover lifelong exposure (70 years), toxicological data are only available for short-term exposure, and it cannot be ruled out that the effect increases over time. An analysis of toxicological studies resulted in the following relations as compared to the traditional, commonly used default factor of 10 mentioned above.

For extrapolation *from subchronic to chronic*, a factor of 10 presumably covers a high percentile of the examined cases (substances) (with geometric means observed in empirical investigations in a range of 2 to 3). This means that for a large number of substances, this factor is sufficient to cover possible increases in effect over time. In terms of the geometric mean, a factor of about 2 or 3 results.

For extrapolation *from short-term ("subacute") to chronic*, a duration extrapolation factor of 4 to 6 appears to be justified, based on the geometric mean.

The factor resulting from such an analysis does not directly represent a measure of the actual dose-time relationship but only describes the commonly observed dose-time relationship, since the design of the respective studies has a strong influence on the outcome (▶ Chap. 9, "Examination of Acute and Repeated-Dose Toxicity").

Extrapolation from LOAEL to NAEL

This extrapolation is applied sometimes in cases where a no observed adverse effect concentration is the desired level of protection and no dose without an effect was obtained in experimental studies.

The data on LOAEL-NOAEL relations reported in the literature reflect the study-design-dependent spacing between doses more than the actual steepness of the dose-response curve. Results will vary depending on the conventions on which the underlying data are based. Therefore, as a better alternative to this extrapolation, benchmark procedures (benchmark dose approach, BMD) are recommended (▶ Chap. 26, "Benchmark Dose Approach in Regulatory Toxicology").

Interspecies Extrapolation

The toxicity of a substance is determined not only by its dose but also by anatomical features and physiological parameters. The relationship between these parameters and body weight, which is used as dose reference, has been observed to follow certain laws (allometric scaling) which deviate from a simple linear correlation. Thus, the basal caloric demand of species of different sizes correlates with the results of toxicokinetic and toxicity (including toxicodynamics) studies in different species. Basal caloric demand correlates with body weight to the power of 0.75.

Consequently, the analysis of relevant available data leads to factors which are bodyweight-dependent and therefore species-specific (scaling factors) for equipotent

extrapolation of data from animal experiments to humans (dose extrapolation based on basal caloric demand (or metabolic rate) scaling). The factors and underlying assumptions are shown in Table 2.

Assuming the allometric relation with basal caloric demand is valid, identical inhalation exposure concentrations must be considered to be equipotent in different species. Hence, no extrapolation factor is applied for this route, or the factor is 1 for all species comparisons.

Of course, individual substances may deviate from this "average situation," leading to higher or lower susceptibility of humans, compared to that predicted by caloric demand scaling. So, if the variability of substance data is to be accounted for, this needs to be considered by an extra factor.

Intraspecies Extrapolation

Sensitivity differences between individuals may be influenced by age, health status, gender, genetic factors (enzyme polymorphisms), or their specific constitution and situation (weight, body mass index, gravidity).

The analysis of available data shows that the commonly used default factor of 10 is probably sufficient to protect a large part of a group of healthy adults, including with regard to potential toxicokinetics-related differences. However, there is considerable uncertainty when it comes to assessing the significance of genetic polymorphisms of xenobiotic-metabolizing enzymes. Data analyses suggest that, in fact, such polymorphisms may lead to large individual differences in internal exposure. When higher internal exposure due to polymorphisms occurs in subgroups with higher susceptibility, such as children, the sick and the elderly, a factor of 10 may not adequately account for these differences, but this variability cannot yet be quantified.

A more in-depth analysis for the group "children" reveals a higher sensitivity, above that of the average healthy adult, of infants, and of new-born babies due to their still-incomplete capacity to excrete xenobiotics. The aforementioned default factor covers this deviation for the most part. In contrast, older children are not considered more sensitive compared to adults with respect to toxicokinetic differences. Phases during which sensitivity is particularly elevated occur in particular during the period of organ development (perinatal exposure) and rapid organ growth.

Consideration of Qualitative Aspects

A distinction must be drawn between the extrapolations addressed above and the consideration of qualitative aspects. The need for this is often justified by qualitative risk assessment aspects. Their quantification requires subjective assessment steps which cannot be scientifically or empirically validated.

Such safety factors are derived to account for, e.g.,

- Data quality (additional factor due to a poor database)
- The quality/severity of the observed effects (additional factor for particularly critical toxicological endpoints; ▶ Chap. 43, "Adverse Effects Versus Non-adverse Effects in Toxicology")
- Grounds for suspicion (additional factor for hitherto unquantifiable potential properties of a substance, e.g., suspected carcinogenicity).

Conventions on this have been defined by, e.g., the WHO and the US EPA (Table 3). The WHO applies a factor of up to 10 to account for suspected carcinogenicity, and the EPA gives a "modifying factor" of up to 10 in case of a poor database. Typically, these factors cannot be validated by data analysis.

Application Framework

The assessment steps discussed here always constitute the attempt to incorporate into the assessment fundamental findings and standards on which no substance-specific knowledge exists. An overview of factors applied by different organizations, and their sizes, is provided in Table 3.

In the case of extrapolations, knowledge drawn from experience can provide justification both for each factor itself and for its quantification. The range this allows to be delimited, or default factors, should be refined as better knowledge becomes available. When in doubt, the decision should generally be in favor of the risk group to be protected. The consideration of better knowledge also means consideration of better, alternative procedures. The replacement of LOAEL/NAEL extrapolation by benchmark procedures is a case in point. For interspecies extrapolation, for example, this means, in the first instance, use of a validated PBPK model (▶ Chap. 29, "Toxicokinetic Models"); in the second instance, use of substance-specific data for species comparison; and in the third instance, an extrapolation based on metabolic rate scaling which also takes data variability into account. Similarly, in risk assessment, valid human data should always be preferred over data from animal experiments, which require additional extrapolation steps (e.g., for differences in susceptibility between species). Most of the existing extrapolation concepts combine the various subfactors by multiplication. However, this is only statistically correct if the individual factors are independent of each other. This is not necessarily the case. For example, when data from a large lifetime animal study are transferred to humans, the age component of sensitive groups of persons may already be covered, at least partially, by the study design. It seems appropriate to limit the size of overall factors obtained by multiplication, for when above a certain level, they express a data uncertainty which makes the performance of a quantitative risk assessment difficult to justify (Table 3). Here too, the use of better alternative procedures, such as probabilistic methods, should be considered where possible (▶ Chap. 24, "Probabilistic Methods in Toxicology"). Recently, a probabilistic framework for hazard assessment was proposed by WHO (WHO 2017; Chiu and Slob 2015).

Regulatory toxicology is concerned essentially with predicting health effects and making decisions on the basis of limited data. In that sense, risks assessment outcomes contain uncertainties, due also to the use of extrapolations and factors, which are problems intrinsic to this field. These uncertainties must be described clearly in order to characterize the reliability of a risk assessment, especially since that reliability is an important information item for risk management.

Cross-References

- ▶ Benchmark Dose Approach in Regulatory Toxicology
- ▶ Dose-Response Analysis: Identification of Threshold Levels for Chemicals
- ▶ Dose–Response Relationship and Extrapolation in Toxicology: Mechanistic and Statistical Considerations
- ▶ Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds
- ▶ Probabilistic Methods in Toxicology
- ▶ Sensitive Humans Versus Average Persons in Toxicology
- ▶ Susceptible Population Groups in Regulatory Toxicology

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Assessment of Background Exposure and Additional Exposure by Human Biomonitoring

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Thomas Göen and Hans-Günter Neumann

Contents

Abstract

Human biomonitoring means the analytical measurement of xenobiotics, their metabolites, and their reaction products (internal exposure monitoring), but also detection of specific effects in human biological molecules and materials (effect monitoring). Its purpose is the evaluation of the exposure, but also of the health risk associated with exposure, by referring to appropriate assessment values. The weight of human biomonitoring parameters is different from case to case, and depends on the linkage of the parameter with the toxic effect, e.g., xenobiotics adducts at proteins and deoxyribonucleic acid (DNA) may be linked more strongly with the carcinogenic risk compared to urinary excreted metabolites. Human biomonitoring is also able to reveal a background exposure of individuals

T. Göen (✉)
Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany
e-mail: thomas.goeen@fau.de

H.-G. Neumann
Department of Toxicology, University of Würzburg, Würzburg, Germany

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without any identified specific exposure, which may result from various environmental sources but in some cases also endogenously from physiological processes. Independent of the source, a quantified internal exposure needs an evaluation, preferably by referring to health-based assessmfent values. In the case of nonderivability of such values, the comparison of human biomonitoring results with so-called reference values or risk-related assessment values may serve an adequate option.

Keywords

Human biomonitoring · Hazardous substances · Xenobiotics · Metabolites · Exposure assessment · Risk assessment · Carcinogens

Introduction

Human biomonitoring (HBM) is used since many decades for the assessment of occupational exposure to hazardous chemicals. A joint statement of the Commission of the European Union and the US occupational safety agencies NIOSH and OSHA defined human biomonitoring as the measurement and assessment of working agents or their metabolites either in tissues, secreta, excreta, expired air, or any combination of these to evaluate exposure and health risk compared to an appropriate reference (Berlin et al. 1982). However, human biomonitoring is not anymore restricted to workplace control, but increasingly includes suspected environmental exposures in individuals of the general population (Boogaard et al. 2011).

Moreover, many human biomonitoring parameters may show already background levels, if they are detected in populations without known specific exposure to the chemical. These may be the result of various environmental sources and exposures and can in some cases also be generated endogenously from physiological processes. This means that background exposure might be composed of avoidable and non-avoidable sources. Food and lifestyle predominantly contribute to background levels. Nevertheless, an additional specific exposure in the proximate environment can affect the internal exposure of the inhabitants too. Every biomonitoring result has to be assessed keeping in mind a possible background and the determinants, which modulate the background (Göen et al. 2012). The additional burden of a suspected compound should therefore be treated as an increment to background (Sugimura et al. 2000).

Human biomonitoring data do not only distinguish between background and additional exposure, but, where reliable associated data on substance-related adverse effects are available, also need a toxicological evaluation. The so-called "human biomonitoring" (HBM) assessment values provide limits, above which health effects cannot be excluded or are probable. Such values are presently generated in many countries around and by international and national researchers, committees, and governmental agencies. The eminent scientific committees for the evaluation and definition of biological assessment values include the Biological Exposure Indices (BEI) committee of the American Conference of Governmental Industrial Hygienists (ACGIH), the

Permanent Senate Commission of the Deutsche Forschungsgemeinschaft for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK Commission), the Human Biomonitoring Commission of the German Federal Environment Agency (HBM Commission), and the former Scientific Committee on Occupational Exposure Limits (SCOEL) of the European Commission. Governmental agencies provide additional assessment values, particularly for questions, which cannot be cleared on scientific basis, e.g., setting of socially accepted risk limits.

Special Issues of HBM Data Assessment for Carcinogens

A special challenge is the evaluation of human biomonitoring data of carcinogenic substances. First of all, it should be considered that substances with carcinogenic potential may differ with respect to their carcinogenic evidence, mode of action, and potency. In this context, the MAK Commission extended the classification of carcinogenic chemicals to five categories (DFG 2020; Neumann et al. 1997). Carcinogens were classified basically according to the strength of evidence: (category 1) sufficient evidence of carcinogenicity in humans, (category 2) evidence of carcinogenicity only in experimental animals, and (category 3) the evidence is inadequate in humans and inadequate or limited in experimental animals, but suspicious data exist. This approach decided essentially about the evidence of the carcinogenic potential, but not about the potency. With two additional categories, the mode of action and the carcinogenic potency were introduced into the classification system. A carcinogenic chemical may either be nongenotoxic (category 4) or genotoxic (category 5), and members of both categories are characterized by having low or manageable carcinogenic potency. The novel perspective indicated by category 5 is that the limit, i.e., a tolerable risk, is not expressed as an absolute value, but based on the contribution to total risk by the active agent (including background exposure and/or physiological background). Low risk means the tolerable exposure does not contribute appreciably to cancer risk.

The distinction between different carcinogens by mode of action and non-appreciable effectivity enables a differentiated assessment of human biomonitoring results. Whereas carcinogens of categories 1 and 2 do not exhibit any clear threshold for avoiding any carcinogenic effect, health-based assessment values, containing prevention against carcinogenic effects, can be derived and applied for substances of categories 4 and 5 (DFG 2020).

Health-Based Assessment Values

The most effective health assessment values are gained by dose–response relationships between internal concentration or dose and the associated adverse effects (No Observed Adverse Effect Level (NOAEL), Lowest Observed Adverse Effect Level (LOAEL)). Those health-based values may be derived from studies, in which both human biomonitoring data and health effects were assessed concurrently. However,

data of human studies are rare and therefore health-based assessment values are often derived from animal toxicological studies. Here, the inequality of the species as well as of the exposure scenarios has to be considered. This is performed by the application of specific as well as generic uncertainty factors. The risk observed with the high doses and the limited exposure periods used in animal experiments must be extrapolated down to low doses and long exposure periods, relevant in the human situation, assuming that the same mode of action works in both cases. Moreover, both the species specificity and individual susceptibility have to be accounted for. The use of default values, like 10 for dose uncertainty, 10 for species differences, and 10 for individual variability, is very common, but may not always meet the real substance- and species-specific conditions.

Moreover, it should be considered that a single substance can provide several health assessment values, each representing a different endpoint. In the field of occupational health, assessment values usually refer to the average "healthy" worker of working age. In this field, uncertainty factors that would protect sensitive persons are usually not applied. Deviating from that, the DNELs (derived no effect levels) under the European "REACH" regulations and the biological assessment values for the general population uses a number of uncertainty factors, to take into account inter-individual variabilities in the average population, with the aim to protect sensitive individuals. Another part of the difference is attributed to the fact that in the field of occupational health, assessment values are based upon chronic exposure over the working life (assuming a total of 40 years and 40 working hours a week), whereas assessment values for the general population in the context of environmental health assumes exposures throughout an entire lifetime (24 h a day, 365 days a year, 70 years). Health-based assessment values in the occupational field, released by the MAK Commission, include the biological tolerance values (BAT) and biological guidance values (BLW). Moreover, there are the biological limit values (BLV) set by the EU Scientific Committee on Occupational Exposure Limits (SCOEL) as well as the biological exposure indices (BEI) published by the American Conference of Governmental Industrial Hygienists (ACGIH).

For environmental health purposes, the HBM Commission establishes the so-called "Human Biomonitoring Values I and II" (HBM I, HBM II). The HBM I value marks the concentration of a chemical in a body medium below which no harm is expected. If the concentration exceeds the HBM I value, further controls are necessary. The HBM II value indicates the limit above which an individual may suffer health effects. In this case, environmental follow-up is recommended and if possible measures should be initiated to reduce the exposure. The HBM I value is considered a check and control value, the HBM II value an interference or interaction point. HBM I/II values are derived either based on human data (approach 1) or a defined tolerable intake (approach 2) or a point of departure for critical effects seen in animal studies (approach 3) (Apel et al. 2017). In the second approach HBM I values are derived on the basis of a daily intake reference value, e.g., acceptable daily intake (ADI), tolerable daily intake (TDI), and reference dose (RfD), which is toxicologically evaluated by scientific commissions, e.g., WHO, US EPA, and EFSA, and already includes the uncertainty factors (see above) (Barnes and Dourson 1988;

Herrman and Yones 1999). Hence, a prerequisite for this approach is the availability of reliable information on the toxicokinetics in humans, which is needed for transferring the daily intake into a level of a human biomonitoring parameter.

Risk-Based Assessment Values

As described, no safe threshold concentration or dose can be specified for genotoxic carcinogens. For these substances an evaluation of HBM results is possibly based on risk-related assessment values, which target the limitation of carcinogenic effects. For these effects, stochastic relationships between the exposure level and the cancer risk can usually be established. Frequently, the supplemental cancer risk is expressed as one additional tumor in an exposed population of specific size, e.g., $1{:}10^5$ or $1{:}10^6$. The weight of evidence of human carcinogenicity and quantitative estimates of inhalative and oral exposures can also be characterized by the unit risk and cancer slope factor, respectively. A unit risk is the upper bound of excess lifetime cancer risk estimated to result from continuous exposure to an agent at a concentration of 1 μg/m^3 in air. A cancer slope factor is the upper bound, approximating a 95% confidence limit, of the increased cancer risk from lifetime dietary exposure to an agent; it is usually expressed as a proportion of a population size affected per milligram per kilogram bodyweight per day (US-EPA 2020).

The question which risk should be considered a tolerable risk cannot be answered with scientific arguments. However, using scientifically derived exposure scenarios together with risk comparison tools, the current sociopolitically tolerated or accepted risk levels can serve as a basis for the derivation of critical exposure values, beyond which a risk would be unacceptable. In Germany, the Committee for Hazardous Substances (AGS) of the Federal Ministry of Labor and Social Affairs has developed such a concept for the determination of occupational exposure to carcinogens. For the concentration of some carcinogenic substances in the workplace air, "acceptance values" and "tolerance values" were defined in the AGS document TRGS 910. The values correspond to an "acceptance risk" defined as an additional cancer risk of 4:10,000 until the year 2018 and 4:100,000 after 2018. An exceedance of a "tolerable risk" of 4:1000 is evaluated as intolerable (AGS 2019). When the quantitative relationships between air exposure and resulting internal exposure is known, the acceptance and tolerance values can apply to results of human biomonitoring, on the basis of exposure equivalents for carcinogenic workplace substances (EKA), compiled since more than 30 years by the MAK Commission for those chemicals at the workplace that have proved to be carcinogenic either in animal or human studies (DFG 2020). The EKA tables deliver the correlation between the workplace air concentration and the resulting internal exposure, when absorption takes place exclusively by inhalation. These correlations allow to derive from the air limit values the internal exposure concentrations, as measured by biomonitoring, that correspond to the acceptance and tolerance risks (see also Göen 2018).

It should be noted that cancer risk values are extremely uncertain. Consequently, no estimate value should be given without the associated uncertainty.

The US National Research Council suggested already in 1974 (National Research Council 1974) that for any single-point estimate, the sources for the calculation and the extent of uncertainty should be stated and the upper bound given. Since this requirement can mostly not be fulfilled, the announcement of point estimates for additional cancer risks is discussed very controversially. A fundamental objection against the unit-risk concept is that the result cannot be tested; it cannot be falsified. With a background cancer rate of 20–25% in the human population, it is impossible to prove an increment of $1{:}10^5$–$1{:}10^6$. The use of default asset values, particularly more than one combined, is quite unsatisfying. It follows that the calculation of an absolute risk leads to uncertain and unrealistic results.

Reference Values and Other Descriptive Assessment Values

There exist other types of descriptive values, which are gained from epidemiological studies in the form of frequency distribution or correlations. A frequency distribution shows how often each obtained measurement value occurs in the studied population. Such frequency distributions often reveal that many substances are detectable in the body of persons, even if there is no specific evidence for any exposure. In such cases, there exists a background internal exposure in the population. The "reference value" reflects the extent of the background exposure. The reference value for a chemical substance in human biological material is a value that is derived from a series of measuring results according to a specified statistical method (Apel et al. 2017; Göen et al. 2012). Samples to be used for this purpose have to be collected from a defined group of the general population (e.g., adults, children, and non-smokers) or the whole population in a randomized way. Data from national environment surveys, for instance, can be considered a suitable base and have been used in several countries to quantify background exposures. To establish a reference value, it is necessary to characterize the reference population and to take care for possible confounding factors as precise as possible. The values are statistically derived from exposure biomonitoring results and are without relevance for health or disease issues. The reference values of a general population are derived from the 95th percentile of a group of persons who are not occupationally or otherwise specifically exposed to the substance. It is often reasonable to define reference values for subgroups, such as smokers and nonsmokers. The 95th percentile value also means that some 5% of the specified group exceed the reference values, which may sometimes lead to discussions. The availability of solid reference values is of great importance as a baseline for the validation of routine- and incidence-related biomonitoring results, both in occupational and environmental context. Reference values may vary between countries, depending on the exposure situations, habits, and hygiene. For Germany, the Human Biomonitoring (HBM) Commission collects and publishes reference values for an increasing number of chemicals, partly

differentiated according to age and gender (Apel et al. 2017). Likewise, the MAK Commission with its mandate to define maximal workplace concentrations evaluates and publishes biological reference values (BAR) (Göen et al. 2012; DFG 2020). Moreover, SCOEL published biological assessment values derived from the background levels in the general population recently too. The latter reference values are of relevance primarily for preventive occupational health care. The results of biomonitoring measurements in the respective class of workers are compared with reference values to find out, if the internal exposure of workers to a substance remains within the background level. Since external exposures to traditional toxicants tended to decrease in the past decades, but those of new substances increased, and confounding habits like smoking changed, reference values need to be reevaluated from time to time (Göen et al. 2012).

As mentioned, there is also a need to correlate external exposures (e.g., inhalation exposure at the workplace) with internal exposure, represented by biomonitoring (HBM) parameters. The exposure equivalents for carcinogenic workplace substances (EKA), for example, describe the relationships between the employee's external exposure (air exposure at the workplace) and the concentration of a HBM parameter (Göen 2018; DFG 2020).

Parameters of Biological and Biochemical Effects

Several biochemical end points exist which correlate with cancer risk and can be used as powerful HBM parameters. This approach basically permits via quantification of reaction products of mutagenic and genotoxic substances, which are covalently bonded as adducts to macromolecules such as proteins (e.g., albumin or hemoglobin) and DNA to estimate risk levels. The experience with biochemical effect monitoring indicates that a contribution to risk could be called non-appreciable if an external exposure leads to an internal exposure and corresponding biochemical effects which are within the variability of the background of a reference population which is not knowingly exposed. If the level of a relevant biomarker lies within the range of the background level, a contribution to risk cannot be established.

Human biomonitoring is not only limited to exposure monitoring but also includes the assessment of effect parameters, which is called biological effect monitoring. However, in this context effect parameters should be of high specificity for the substance or substance group. Biological effect monitoring is closer to the end point of disease than biomonitoring of exposure. Biological effect monitoring provides information about the biologically active dose and the individual susceptibility. The difference to the generally used risk assessment is that the end point of the assessment is not the population-based disease but the use of analytical data from an individual. The data represent the immediate situation of an individual human, without any defaults for the environmental situation or species differences or susceptibility.

Can Thresholds Be Certain?

A threshold is usually understood to separate an effective from an ineffective dose, although one may find in most dose–response relationships an exposure for a biological end point for which no strain can be seen (NOAEL). Even with ineffective exposures to compounds producing reversible effects, the search for the "ineffective threshold" is not anymore adequate. Now it appears more appropriate to look for deviations of physiological balances, which are detectable below the NOAEL, and to assess how well the system adapts against strain and which degree of imbalance should be considered adverse.

The cell adapts to stress at the mitochondrial respiratory chain, for instance, with an increased synthesis of respiratory chain components. If that stress increases beyond a critical point, the cell is eliminated by apoptosis and substituted by a new one. Several signaling pathways are involved and may control a common end point, such as apoptosis (Bolt and Degen 2004). With this perspective, it is not reasonable to search for a concentration threshold of ineffectiveness. It is important to find out how much a certain stress affects the cellular energy balance, in other words, leads to proliferation. A threshold between effect and no effect cannot be defined for a chemical which affects the respiratory chain. Effects on processes taking place below the NOAEL should be considered as stress, which under favorable circumstances can be compared with a reference value. DNA lesions produced by genotoxic compounds have been shown experimentally to be linear down to extremely low doses without threshold. This does not support the threshold concept on the molecular level (Neumann 2009).

Cross-References

- ▶ Do Carcinogens Have a Threshold Dose? The Pros and Cons
- ▶ Dose–Response Relationship and Extrapolation in Toxicology: Mechanistic and Statistical Considerations
- ▶ Exposure Scenarios in Toxicology
- ▶ Human Biomonitoring: Its Importance in Toxicological Regulation
- ▶ Importance of Exposure Level for Toxicological Risk Assessment
- ▶ Uncertainty Analysis in Exposure Assessment-Relevance for Toxicological Risk Assessment

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Adverse Effects Versus Non-adverse Effects in Toxicology

43

Norbert Englert and Robert L. Maynard

Contents

Abstract

The term "adverse" is used with the meaning "disadvantageous" or "harmful." An effect should be avoided if it is adverse but could theoretically be tolerated when non-adverse. Generally it is more or less clear what is meant by "adverse," but in a real situation, it may be difficult to position the line separating adverse from non-adverse.

Keywords

Causality · Detectability · Probability · Reversibility · Severity · Subgroups

N. Englert (✉)
Formerly: Federal Environmental Agency, Berlin, Germany
e-mail: norbert.englert@web.de

R. L. Maynard
Birmingham University, Birmingham, UK

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The Meaning of the Term *Adverse*

The term "adverse" is an adjective which is often used to describe negative effects on health. We take the term "adverse" to imply some degree of harm or the likelihood of unfavorable consequences for the individual or population concerned. The term "undesired" seems, to us, less appropriate as a synonym for "adverse": not all effects that might reasonably be described as undesirable need necessarily be adverse.

The term "adverse" is commonly used without reference to a clear definition. Using imprecisely defined terms in assessments of effects, or possible effects, on health is not at all uncommon. For example, Article 3 of the Charter of Fundamental Rights of the European Union says: "Everyone has the right to respect for his or her physical and mental integrity."

Definition of Adverse Effects

The IPCS/WHO definition of adverse effects, published in *1994* (IPCS/WHO 1994), is often quoted, but the second and very important sentence of the definition (our italicizing below) is frequently omitted:

> "Adverse effect: change in morphology, physiology, growth, development or life span of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increase in susceptibility to the harmful effects of other environmental influences. *Decisions on whether or not any effect is adverse require expert judgement*."

In 1985 and 1999, the American Thoracic Society (ATS) tried to define adversity in context of air pollution. Supplementing their 1985 statement, in 1999 the ATS referred to health-related quality of life. However, the ATS emphasized that this statement does not offer strict rules or numerical criteria, but rather proposes principles which may be used in weighing the evidence and setting boundaries between adverse and non-adverse health effects, and the placement or positioning of dividing lines should be a societal judgment (ATS 1985, 1999). An update of these statements discusses a wider range of effects and biomarkers as a "set of considerations" for judgments on the adversity of effects (Thurston et al. 2017). Any increase in deaths from cardiovascular or respiratory disease is classified as obviously adverse. For some effects or biomarkers the extent of change that the authors consider to be adverse is discussed, and other effects or biomarkers are discussed only under semi-quantitative or qualitative aspects.

In including quality of life and well-being, ATS is supported by the 1948 WHO definition of health (www.who.int/about/definition/en/print.html): "Health is a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity."

Dimensions of Adversity

The ATS statements and other summarizing reviews indicate and define several dimensions or characteristics of adversity, in part referring to an individual concerned, in part, in addition or exclusively to a population, and also referring to qualitative or general aspects.

Dimensions of adversity:

- Severity of an effect
- Detectability of an effect
- Reversibility of an effect when exposure stops
- Probability of an effect
- Particularly concerned subgroups ("environmental justice") and effect on current or next generation
- Causal relationship between exposure and effect

Severity of an Effect

According to ATS, death or an increased risk of death and any consequence on life expectancy as well as clinically significant effects should be classified as adverse effects.

For respiratory symptoms and changes in indices of physiological function, the ATS sees the line between non-adverse and adverse being crossed if quality of life is impaired. In this context, health-related quality of life refers to factors including capacity to look after oneself, mental health, pain, and generally feeling well. Decrease in health-related quality of life is classified as adverse, e.g., shown by clinically significant findings when diagnostic tools, including questionnaires, are used to assess health status.

Keeping in mind that there is a considerable lack of knowledge of the meaning of changes in biomarkers, ATS does not generally classify changes in biomarkers as being adverse. The 2017 statement offers advice for assessing adversity of changes in biomarkers.

Detectability

Concerning detectability of effects, statistical significance is usually demanded. Tests of statistical significance are used to distinguish, with a conventionally acceptable but arbitrary level of confidence, between effects that might be taken to imply some actual relationship between a potential cause and an effect and those which might occur by chance. Thus, unless an association reaches "statistical significance," it is likely to be ignored. The possibility of error should be noted. Large epidemiological studies have great statistical power and can detect very small effects. Thus, in the air pollution field, a change in the daily average

concentration of particles defined by PM_{10} has been shown to be associated with a less than 1% increase in the risk of death over a short period. This is a small effect but the coefficient that specifies the effect is statistically significant and is thus regarded as unlikely to have resulted from chance.

Statistical significance should not be taken as proof of a causal relationship: the possibility of confounding needs to be considered. Death is clearly an adverse effect on health, but is a small increase in the risk of death also, by definition, an adverse effect? It should be noted that when death is discussed, the long-term risk, for all, is absolute: we all die. What is usually meant is an increased likelihood of death in a specified period or a shortening of life expectancy. This opens up the question of what degree of shortening of life expectancy should be regarded as adverse. Many would say that any effect on life expectancy is an adverse effect. This leads to a rather philosophical point: the effect may be adverse but is it important? There is no scientific way of answering this question: the answer will vary from person to person.

In the 1985 statement, the ATS showed a pyramid or triangle to illustrate the relationship between severity of an effect and number of persons likely to be affected. At the top of the pyramid is death as the most severe effect, followed by disease, and finally respiratory symptoms. This pyramid, broadening from top to bottom, is thought to illustrate that with decreasing severity the frequency of an effect increases in a population. The hypothesis that influences on mortality are severe but rare effects, however, no longer corresponds to current knowledge at least with respect to particulate matter. It seems reasonable to think that less exposure to some toxic material is needed to produce a minor effect than a major effect, but work in the air pollution field has shown, especially with regard to particles, that at all ambient concentrations studied, effects on all outcomes (deaths, hospital admissions, symptoms, restriction of daily activity) occur. Thus, it is now felt that the idea of different thresholds separating exposures with effects on, for example, symptoms from exposures with effects on the likelihood of death is incorrect. Whether or not a very small decrease in life expectancy should really be seen among the most severe health effects may be debated, but we can no longer assume that effects on mortality generally concern only a very small part of the population exposed.

The traditional classification distinguishing between substances without effect threshold (carcinogenic substances) and those with effect threshold is not supported by recent findings. This makes the classification of toxicologically active materials by their effects more difficult. Without an effect threshold, a purely qualitative statement (effect/no effect) is no longer adequate. There will always be the quantitative aspect to be added (i.e., how large or how frequent an effect may be or should not be).

Subjective Perception

At an individual level, the more an effect is perceived as imposing a limitation on physical or mental activity and as having negative emotional overtones, the more the effect is likely to be classified as adverse. Aspects including the assessment of the

possibility that action taken by individuals can influence effects or – in contrast – perceptions of helplessness can act as modifiers.

The subjectively perceived probability of an effect occurring at a certain exposure does not always correspond to "objective" reality, but it contributes to the subjective assessment of adversity. A feeling of threat and danger subjectively associated with environmental influences might also be an adverse effect – beyond the influence of environmental regulators.

Reversibility

Reversibility of effects after the end of exposure is certainly very important as regards symptoms and functional changes. Complete or partial irreversibility would suggest adversity (the occurrence of adverse effects); complete reversibility might support non-adversity, especially in the case of very low severity of effects. However, an effect which is reversible for most of the persons exposed might be irreversible for susceptible individuals. With respect to functional physiological parameters, the ATS advises that minor transient deteriorations of lung function values should not to be automatically classified as adverse, but if connected with symptoms, they should. A detectable, permanent deterioration of lung function, however, is classified as always adverse.

Probability and Number of Persons Concerned

In terms of clinical parameters, at a population level even a minor level of effects of air pollutants is generally declared unacceptable by the ATS. This may be an example of the fact that changes classified as being not adverse at an individual level may demand different consideration if they occur in a group or a population. Figure 1 schematically shows that a possible classification as adverse or non-adverse depends on the severity of the effect in an individual concerned as well as on the percentage of persons concerned in a population at a certain exposure.

Below the line through y, the severity of effect in an individual concerned is so low that the effect would not be classified as being adverse even if the whole population were to be affected. Left to the line through x, the number of individuals concerned is so low that a relationship to exposure is not verifiable due to statistical reasons. To the left of and below the dashed curved red line, an effect is not adverse because the severity of the effect is small even considering the number of persons concerned. To the right and above the dashed curved line, an effect is classified as adverse because considering the severity in an individual concerned as well as the number of persons concerned in the population exposed seems to be not tolerable. In this case, the large fraction of persons concerned suggests the classification of an effect as adverse even if it would be classified as non-adverse at an individual level.

However, the difficulties begin when one tries to fix the positions of the lines x and y on defined and scaled axes.

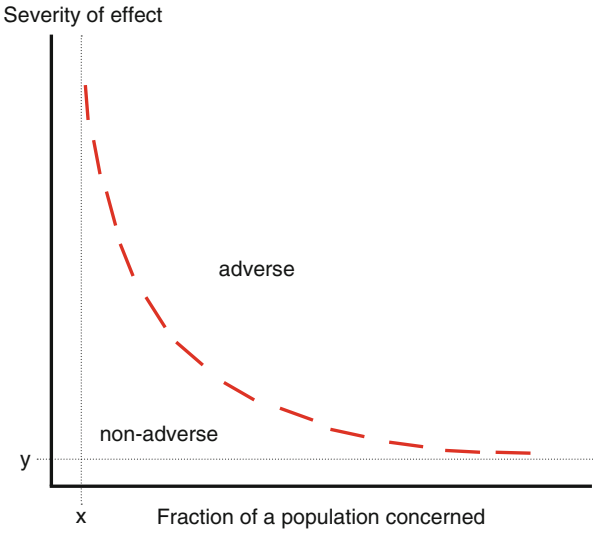

Fig. 1 Pragmatic classification of an effect as adverse in relation to the number of persons concerned and to the severity of the effect in concerned individuals

Groups Concerned

If effects are limited to certain subgroups of the population, this will need to be considered when assessing effects at both an individual and population level. Effects focused on certain subgroups may lead to different assessments on an individual level ("I am not concerned, so it is not so severe") and at the level of effects on public health ("it is a particular injustice because some groups are at a much higher risk"). Moreover, the severity of an effect may be neglectable in more or less healthy people but important in frail or impaired persons.

Effects probably appearing for the first time in the next generation tend to be classified as "adverse," although due to the potential manifestation in the future, the uncertainties are larger than in effects without large temporal latency.

Causality

The question of causality is a matter of discussion in all epidemiological findings. The Bradford Hill features of causal associations may be helpful (Hill 1965), but whether or not an effect – be it directly measured or "only" calculated – is attributed to its real cause cannot always be clearly decided.

Whether or not a causal chain: *cause – change in a biomarker of exposure – change in a biomarker of effect – functional change – symptom – disease – death* could be demonstrated or only hypothesized and whether that chain describes a possible or an unavoidable course should be considered when assessing the adversity or non-adversity of the initial steps of such a known or hypothesized chain. And an additional aspect may be the possible "co-operation" of causes which are not powerful alone but effective in combination.

Final Remarks

Generally, the IPCS/WHO definition of 1994 – extended by aspects of (subjectively perceived) quality of life – seems to be a reasonable basis for deciding on whether an effect should be regarded as "adverse." For classifying an effect as adverse or non-adverse, it is not possible to give precise criteria which in any particular case result in an "objective" and transparent assessment. Expert judgment remains a necessity. This should not be seen as an unavoidable deficiency but rather as an opportunity for a discussion exactly adapted to each particular situation. Which persons are the appropriate experts who should be authorized to perform such a judgment is a question as difficult as that of the threshold between adverse and non-adverse. In a larger context, this is subject to societal agreement.

For regulators, expert advice on adversity of a health effect is a necessary and helpful basis for standard setting. Regulators have to consider aspects including feasibility, juridical implementation, and cost-benefit relations. In all, the ideal of setting standards which avoid any adverse effect on health is an aim which indicates the direction to follow even recognizing that it may never be entirely reached.

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Health-Based Threshold Values Versus MOS in Toxicology

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Ursula Gundert-Remy

Contents

Abstract

The derivation of health-based threshold values in various fields of regulatory toxicology is based on consented rules laid down in regulatory guidance papers (EFSA EFSA J 8:1325, 2010; ECHA, 2010) Guidance on information requirements and chemical safety assessment Chap. R.8: characterization of dose [concentration]-response for human health ECHA-2010-G-19-EN). The rules were developed according to the field of application and are improved when scientific evidence became available showing that elements of the framework have to be changed. No principle difference exists in the guidances from several European agencies.

The MOS concept applies the same principles; it is however more flexible as it allows to introduce scientific judgment.

Keywords

Health-based threshold values · Assessment factors

U. Gundert-Remy (✉)
Institute for Clinical Pharmacology and Toxicology/Institut für Klinische Pharmakologie und Toxikologie, Charité Universitätsmedizin Berlin, Berlin, Germany
e-mail: ursula.gundert-remy@charite.de

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Introduction

In toxicology, risk assessment requires quantification of: (1) the hazard given by the inherent toxicological properties of a substance (resulting from experiments in animals or epidemiological data) and its dose-response characterization and (2) the exposure in quantified terms. Comparing the exposure with the outcome of the hazard assessment (a dose which is assumed not to exert adverse effects) gives the answer to the question "how dangerous for health is the exposure towards this substance?" This comparison is called risk assessment. Two approaches are used in regulatory risk assessment of chemicals. One approach is to set health-based guidance values, such as accepted daily intake (ADI) or tolerated daily intake (TDI), and to calculate the ratio between exposure and health based guidance value. If the value is below 1, that is, the exposure is smaller than the health based guidance value the outcome of the assessment is "no concerns." The second (margin of safety, MOS) approach assesses the distance between the exposure and the dose without an appreciable effect. In general, it is assumed that the exposure is of no concern if the exposure is 100 fold lower than the dose without an appreciable effect. Depending on the data set available and the specificity of the substance, a higher factor than 100 may deemed necessary.

Health-Based Threshold Values

The basic concept of health-based threshold values, such as ADI values (acceptable daily intake), was introduced by JECFA (Joint Food and Agriculture Organization – World Health Organization Expert Committee on Food Additives) already in the 1950s. Applying the evaluation scheme, food additives are assessed by an international panel of experts. Later, the concept was adopted by the Joint Food and Agriculture Organization – World Health Organization Meetings on Pesticide Residues (JMPR). In the beginning, ADI values were developed only by the two scientific panels. Later, when the European Food Agency (EFSA) was established in 2002, ADI values were derived by scientific panels working for EFSA.

Other European regulatory bodies, in particular the European Chemicals Agency (ECHA), also used the concept to derive so-called derived no-effect levels (DNELs) whereby some differences exist between the two European agencies as EFSA derives a single ADI, whereas ECHA derives several DNELs depending on the route of exposure, even for specific end points separately, and separate DNELs for healthy workers and for the general population.

The principle of the concept is to define a dose, which, based on the scientific evidence, can be assumed to be safe, that is, without adverse health effects in humans even if the dose is taken up on a daily basis for a lifetime. In most of the cases, the point of departure (POD) to derive a safe dose is experimental data from toxicological studies in animals. The dose which did not produce an adverse effect in animals (no observed adverse effect level or NOAEL, lower benchmark dose level or BMDL) serves as the POD. The dose is adjusted to the human situation by

using a factor accounting for the interspecies difference and a second factor accounting for the variability in the human population to derive the dose which is assumed to be safe during a daily lifelong exposure by food (ADI), dermal contact, and inhalation (DNEL). Knowing this dose allows regulating exposure at a level which will not result in adverse health effects in the exposed population.

Dose Without (Adverse) Effect

The dose without an (adverse) effect is the dose for which, in an experimental study, no effect has been observed compared to the control treatment. It should be taken into consideration that the dose critically depends on the experimental conditions such as number of animal tested, dose range and dose interval, and the range of end points tested (e.g., histopathology, clinical chemistry, and functional tests). It has not yet been decided whether the existence of an effect has to be demonstrated by a statistically significant difference compared to control or whether biological plausibility is sufficient.

The limitation of the procedure on how to derive a dose without effect is obvious. We must determine a dose with an adverse effect in order to be able to define the neighboring lower dose as the dose without an effect. Hence, the number of animals and the spacing of doses are crucial in this respect. If we do not see an effect, this is not proof for absence of an effect. Animal protection requires reducing the number of animals, whereas statistical power considerations would require a high number of animals. We should be aware that OECD guidelines are a compromise between the two principles which does not prevent us in overlooking effects because of the low statistical power of the study.

It has to be discussed whether the point of departure (POD) to derive health-based threshold values is a no-effect level (NEL) irrespective if the effect is adverse or adaptive or compensatory or just a biochemical change. In particular, in the era of genomics, studies at the level of genes do allow to observe changes which, however, cannot be attributed to be adverse. NAEL is used to describe a theoretical no-adverse-effect level, and NOAEL is the not-observed-adverse-effect level. An example for an adaptive effect is the induction of drug metabolizing enzymes in the endoplasmic reticulum in the liver, which will lead to an enhanced metabolic capacity and thus a reduced toxicity if the parent compound is the toxicant. An example for compensatory changes is the increased inhalation rate in metabolic acidosis. In the International Program on Chemical Safety (IPCS/WHO), definition of adverse effect is as follows: change in the morphology, physiology, growth, development, reproduction, or life span of an organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences. Today, the NOAEL is taken as POD to derive health-based threshold values.

The duration of the study is also influencing the level of the NOAEL: this is partly due to the fact that in subacute and subchronic studies, the number of animals and also the number of parameters investigated are lower than in chronic studies which

Table 1 Cumulation factors for substances with long half-lives: 28 day study versus 90 day study

Half-life (days)	% of steady state reached after 28 days	% of steady state reached after 90 days	Accumulation factor
7	93.75	100	1.07
14	75	100	1.3
28	50	90	1.8

may lead to higher NOAELs in subacute and subchronic studies as compared to chronic studies. Sometimes, adverse effects may only be developing after long-term exposure. One explanation can be the kinetics. Substances with long half-life will cumulate and reach the maximum level only after prolonged exposure as demonstrated in Table 1.

Toxic effects may develop only after prolonged exposure, for example, effects on the testes or thyroid as secondary effects with a primary effect on the liver. It is currently becoming clear that the whole database has to be taken into account including the studies for developmental toxicity and fertility. Those studies produce additional information not seen in repeated dose testing.

It is to be noted that using NOAEL as the POD does not use all available information on the dose–response relationship. International agencies (e.g., IPCS–WHO, EFSA) have therefore given advice to use all information by modeling the dose–response relationship and using the curve to derive a dose which corresponds to a low effect level. This is called the Benchmark dose approach. The Benchmark dose (BMD) is a dose level, derived from the estimated dose–response curve, associated with a predefined change in response, the Benchmark response (BMR) which size is to be determined based on the variability of the selected endpoint (Slob 2017). Several mathematical formulas exist for the modeling of the experimental data. In the most recent version of applying the Benchmark dose approach, it is recommended not to select a specific formula but to use a model averaging approach (EFSA Scientific Committee et al. 2017). For risk assessment purposes by convention, the lower confidence interval of the BMD, the BMDL, is used.

Safety Factors/Uncertainty Factors/Assessment Factors

Point of departure (POD) for the derivation of a health-based threshold value is the NOEAL/the Benchmark dose from a chronic dose in animals, often in the rat. Adjustment factors are used to "adjust" the dose in the rat to the respective dose in man. It is assumed that in general the human organism is more susceptible when compared to the rat. To bridge the species difference between rat and man, a factor of 10 is used. The interspecies factor is subdivided into a factor accounting for the differences in toxicokinetics and a factor accounting for differences in toxicodynamics. The toxicodynamic factor is 2.5, a value which is not well supported by data. The toxicokinetic factor is dependent on the species and based on allometric considerations. For the rat, the factor is 4, rendering the total interspecies factor to 10. For mice the toxicokinetic factor is 7, rendering the total

interspecies factor to 17.5. For rabbit the toxicokinetic factor is 2, rendering the total interspecies factor to 5. The factors can be modified (so-called chemical-specific adjustment factors) if chemical-specific scientific data are available (WHO 2005).

An additional factor is used to account for the variability within the human population. The intraspecies factor is subdivided into a factor accounting for the toxicokinetic variability and a factor accounting for the toxicodynamic variability. The default value which is used is 10 whereby a factor of 3.14 accounts for toxicokinetics and a factor of 3.14 for toxicodynamics. Data from clinical studies showed when retrospectively analyzed that the factor of 10 is empirically supported. Only with chemicals/drugs metabolized by polymorphically expressed CYPs (such as CYP 2D6), the factor of 10 is not appropriate and a much larger factor is needed. If chemical-specific data are available, it is advised to use the data-derived factors instead of the default value ("chemical-specific factors"). International experience, increasing utility, and regulatory acceptance of chemical-specific factors have been described in a recent publication (Bhat et al. 2017).

Margin of Safety (MOS)/Margin of Exposure (MOE)

There are situations where human exposure occurs, but no guideline level (such as ADI or TDI) is available that would help to assess the health risk of the chemical. In such circumstances, the value for the NOAEL in the available study is divided by the exposure level. The quotient is called the MOS or MOE (in cases of genotoxic carcinogens). The margin of safety is also used to assess the health impact in cases in which the exposure is higher than the ADI/TDI. The following aspects are to be taken into consideration when assessing the MOS/MOE: (1) scientific rigor of the database, (2) possible difference in the route of exposure between animal and man, (3) differences in the exposure scenario between animal experiment and human situation (duration, dosing, frequency of dosing: very often the total daily dose is given in one dosing, whereas in humans the dose might be divided in three meals), (4) steepness of dose–response relationship, (5) nature and severity of the effect, (6) differences between species, and (7) variability in the human population including possible sensitive subgroups. There is presently no regulatory document discussing cases explaining the derivation of an appropriate MOS.

Exposure Assessment

It should be mentioned here that the assessment of exposure is as important as the hazard identification and dose–response assessment. The first step is the identification of the appropriate scenario, the second the parameterization of the scenario. Whereas for food intake databases have been collated and the content of chemicals in food is well known for most of the food items, the exposure situation is far from being known for other substances and other circumstances such as consumer products. In order to take a cautious approach, "worst case" assumptions are made which bear the potential to grossly overestimate the exposure.

Risk Assessment

If the exposure is lower than the health-based threshold, it can be concluded that there is no health concern. Concerning the MOS/MOE a factor of 100–300 is sufficient for noncarcinogens and nongenotoxic carcinogens depending on the database. For genotoxic carcinogens, a MOE greater than 10,000 is interpreted in the way that no urgent measures have to be taken (EFSA 2012). In cases where the exposure is higher than the health-based threshold value, the MOS approach can be informative to assess the possible health impairment. For example, given the same effect (e.g., hepatotoxicity), a MOS of 10 is of higher urgency for measures as compared with a MOS of 90.

Cross-References

- ▶ Assessment of Limit Values in Regulatory Toxicology
- ▶ Benchmark Dose Approach in Regulatory Toxicology
- ▶ Uncertainty Analysis in Exposure Assessment-Relevance for Toxicological Risk Assessment

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Precaution Principle Versus Danger Prevention in Toxicology

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Abstract

The pollution of water, soil, air, food, and everyday products with harmful chemicals is accompanied by risks for public health. The active defense or control of these risks can be effected using the principles of hazard prevention or precaution, respectively. Toxicological information is a basic contributor to preventing and controlling hazards together with data from other disciplines.

Protection of the health of consumers and their environment is dependent on scientific information and associated policy with preservation of public health through various measures. More specifically, consumers of food and everyday products are afforded protection by official regulations which concentrate on surveillance of the market and manufacturers and warnings against goods recognized as presenting actual or potential adverse effects for the public.

L. Müller (✉)
Formerly: State Health Administration, unit 44 (Pharmaceutics, Toxicology, Genetic Engineering), Bremen, Germany
e-mail: lumuebas@aol.com

N. H. Stacey
Southern Cross Pharma Pty Ltd, Malua Bay, NSW, Australia
e-mail: nstacey@southernxp.com

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Environment-related health protection aims at safeguarding the public from toxic (carcinogenic, genome-altering, and other) effects that may come from contaminated water, soil, and air. Necessary tasks include the recognition and description of environmental influences adverse to health, the prevention or the removal of these influences where applicable, and the development and the transfer/mediation of findings such that harmful inputs from the environment may be avoided.

The administrative regulation of substance-related risks in this field is based on hazard prevention and/or precaution.

Principle of Hazard Prevention

In general law, the term "danger" is described as a situation, which leads to damage of a protected legal good within a reasonable timeframe and with sufficient probability, if the expected course of events is not stopped. The basis is a safe prediction of the course of events. The requirements for the indication of danger are less demanding if the legal good in question is highly ranked (e.g., human life) and if serious damage is expected (e.g., health damage). The prevention of the development of such a situation is called hazard prevention.

To initiate official measures of hazard prevention concerning chemical substances, usually, a numerically fixed minimum triggering level, a threshold of danger or adverse effect is required. Because of the enormous legal consequences, in some cases, pure suggestion of hypothetical damage, 1 day is not sufficient to allow (legal) stipulation of the fixing of an absolute limit. Instead, the threshold must be based on scientific or otherwise obvious knowledge of a particular limit above which human health effects may occur.

The toxicologist may essentially contribute to the characterization of a threshold of danger by:

- Definition of the relevant route(s) of exposure
- Estimation of the extent of exposure
- Characterization of risk groups
- Determination of mechanism of action of a so-called adverse effect: an important health effect of not just a temporary nature out of a range of effects
- Assessment of a dose–response relationship
- Estimation of a NOAEL (no observed adverse effect level) or a NOAEC (no observed adverse effect concentration; for instance, in the context of air quality values) or otherwise derived thresholds of effect (for non-genotoxic substances, for instance, using the benchmark procedure)
- Determination of a limit dose by using assessment algorithm (e.g., unit risks) for genotoxic carcinogens based on "politically" agreed levels of acceptance or tolerance

Such characterization is based on appropriate animal studies (the quality of which is preferably categorized by the so-called Klimisch criteria (Klimisch et al. 1997), epidemiologic findings, and additional reliable data. In some cases, single observations in humans after accidents or disasters/catastrophes may also be of use in this endeavor.

Suspected Threshold of Danger

During toxicological assessment, there are often imponderable aspects that arise due to the lack of useful epidemiological data and the ensuing need to extrapolate animal data (often at high doses) to long-term effects in humans linked to illness (mostly at lower doses relevant in the environment of the affected people). The resulting uncertainties need to be compensated by different (un)certainty and extrapolation factors, such as those proposed by the WHO, the ECHA (2010), or the German Committee on Hazardous Substances (AGS 2010). With respect to legal requirements, in some cases, this is also true for additional so-called danger-linked factors.

The quality of the basis of evaluation, the extent of the broadly agreed factors involved, the experience and the expertise of toxicologists, and finally political considerations influence the determination of the regulatory limit as shown as the area of danger marked in the risk plot, here better described as suspected threshold of danger (Fig. 1). Because of the mainly toxicologically based derivation, the principle of protection by hazard prevention is regarded as a toxicological principle.

Principle of Precaution

The principle of precaution is based on the assumption of a risk continuum. It follows that the possibility of a health risk below the threshold may not necessarily be totally excluded, especially when investigating worst cases with respect to vulnerable groups of people. This is of particular relevance when considering

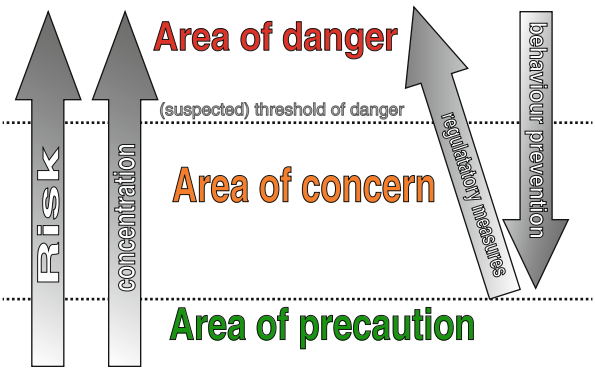

Fig. 1 Assessment areas for harmful substances in the environment

genotoxic carcinogens. However, with the depletion of the concentration of the pollutant in the environmental milieu, the risk declines, and the initiation of health deterioration will become less probable (Fig. 1). The principle of precaution finally aims to reduce this risk to the greatest possible extent.

The application of the precautionary principle is thought to compensate for possible uncertainties (for instance, due to gaps of the toxicological data base) within the evaluation of substances/groups of substances (Mitteilung der Kommission 2000).

One special manifestation of the principle of precaution is the principle of minimization, as found in several legal acts. This means that, for instance, the concentration of chemical substances polluting the environmental source or adversely influencing its quality (e.g., drinking water) has to be kept as low as ultimately possible according to the state of the art while considering the circumstances of the particular case with reasonable expense/effort. In brief, it is referred to the ALATA (as low as technically achievable) or the ALARA principle (as low as reasonably achievable).

The principle of precaution also involves the predictive or forward-looking protection of people against adverse exposure by the development, by recommendations, and by the enforcement or implementation of measures on the basis of health quality goals. This is strongly linked to the term of sustainability, the future viability of quality goals, the results of which satisfy the needs of living people. At the same time, these results should not reduce the chances of future generations for a healthy existence.

The burden of exposure of human beings from environmental sources not only should be minimized but rather should be removed or eliminated wherever possible.

Overall, the principle of precaution therefore incorporates the general aspects of environmental health.

Distinction of the Area of Precaution

In regulatory affairs, it is often necessary to complete such qualitative considerations by quantitative assessments/evaluations to enable administrative/official measures, if needed.

On the scale of a continuum between risk at the (suspected) threshold of danger and (unrealistic) zero risk, in principle, every value may be accepted for the entry into the area of precaution (precaution value). However, usually, the assumption is followed that a small deviation from the (suspected) threshold of danger is not enough to enter the precaution area. Instead, below the danger area, an area of concern is assumed in which individual hazards are not yet excluded. This area is tied to the area of precaution, in which a health hazard does not exist anymore or would be extremely unlikely (Fig. 1).

However, due to a lack of scientifically reliable data, the threshold to the area of precaution cannot be derived just by toxicological methods. Rather, it is oriented on technical, aesthetic, or general aspects of human well-being. Due to these considerations, the largest possible margin to the (derived) thresholds of effects (suspected

threshold of danger) is usually chosen. It is expected that this approach can allow for current imponderables and differences among individuals.

In this context, the toxicologist's expertise is needed with regard to, e.g., information about and weighting of the extent of, for instance, carcinogenic, immunotoxic, neurotoxic, and development blocking effects in the lower risk area, as well as guiding principles for further yet-to-be-fully-resolved exposures, vulnerabilities, and potentials of hazard.

This envisaged level of protection which is beyond the scope of protection against danger is regarded as mainly based on general aspects of environmental health.

This principle manifests itself in the development of (precaution) standards and quality goals with contributions of toxicologists in official working groups, committees, and commissions, for instance, to solve questions with respect to health impact assessment, land use planning, and so on. As for risk management, the implementation of such working results is usually modified to a high degree by the political weighting of various interests.

Along with administrative/regulatory measures, official recommendations and advice are addressed to users and consumers, such as aiming to avoid sources of pollution (behavioral prevention) (Fig. 1).

Cross-References

► Hygienic Versus Toxicological Approaches in Regulation
► Toxicological Risk Assessment

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Hygienic Versus Toxicological Approaches in Regulation

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Roland Suchenwirth and Stephanie Hüser

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Abstract

The hygienic approach aims at keeping the environment and the human body free of avoidable loads, which means to minimize – not necessarily exactly specified – environmental contamination and thus at the same time to prevent chemically induced illnesses. The toxicological approach assumes that an exposure level is defneable – that does not lead to specific adverse health risk – and derives tolerable exposures from this starting point. Both approaches often seem to complement each other in regulatory considerations, as they possess different points of view and aims.

Keywords

Hygiene · Toxicology · Minimization principle · Precaution · Precautionary principle · Prevention · Risk · Drinking water

R. Suchenwirth (✉) · S. Hüser
Department of Environmental Hygiene, Governmental Institute of Public Health of Lower Saxony, Hannover, Germany
e-mail: Roland.Suchenwirth@nlga.niedersachsen.de; Stephanie.Hueser@NLGA.Niedersachsen.de

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Introduction

While toxicology derives tolerable or acceptable doses based on present knowledge (experimental or modelled date), hygiene aims at keeping the environmental media free of pollution/contaminants, which has the effect to reduce and avoid exposures (Fig. 1). The two concepts or disciplines have a right to exist in parallel in the regulations. They use quite different approaches that still reflect the ancient Greek terms "hygiene" (for health, salutary) and toxicon (poison). Today both approaches have a common purpose: protection of the human health, the population and the living nature. The differences are less in the type or content of applied methods, but rather in the way of thinking already from the beginning of a regulatory process.

Schematic drawing showing a log dose versus effect plot. Hygienic precautions are made, even in the absence of any presently detectable risk. Toxicological approaches are interested in the doses, where a threshold is overrun or where danger must be assumed.

Toxicology

The assessment in toxicology is primarily based on the knowledge of effects caused by an individual substance (or a defined group of substances) and is evaluated according the principle defined by Paracelsus "dosis sola facit venenum."

Absolute thresholds without detrimental effects are mostly derived from experiences with (high) toxic doses for a single substance or group of substances under strictly defined conditions (e.g., certain occupation-types or animal species). The definition of strict conditions aims at the reproducibility of results and the decreasing

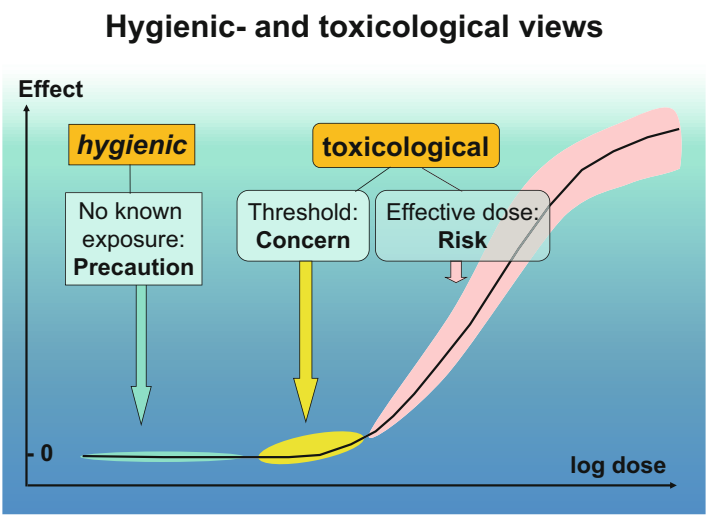

Fig. 1 Hygienic and toxicological view

the impact of confounding factors by minimizing variables. However, obtained results are sometimes difficult to translate to a complex exposure scenario as such of human beings. For example, up to now, most of the occupational exposure limit values (OEL) or also acceptable daily intake values (ADI) are generated in previous described way.

Environmental toxicology has the objective to assess health risks associated with substances of geogenic or anthropogenic nature and their distribution in the environment. The human being as well as the living nature is in its focus. Bioassays and studies with in vitro systems are used to determine or model the physical factors and the toxicity of substances that occur in the environment as intended active ingredients, residues, or resulting contaminants.

Limit values for pollutants in environmental media consider – in comparison with workplace regulations – the potentially longer persistence and exposure (24 h per day, each day of a year) and the higher diversity of influencing conditions, such as the potentially higher sensitivity of special population subgroups. It can be stated, however, that in most areas of regulation (work, environment, etc.), the idea of prevention and the hygienic approach to protection becomes increasingly important.

Hygiene

Hygiene is more far-reaching, multifaceted, but at the same time, it is also more indistinct and less definable: Its scientific and educational approach aims at the prevention and control of illness as well as health preservation in particular through health protection and health promotion. The science of hygiene investigates all illness-causing factors in the natural, technical, occupational, chemical, microbiotic, and social environment. Corresponding to that, the discipline develops counterstrategies and countermeasures to take the impact of these various influences appropriately into account.

Inherent to the vision of hygiene is the "precautionary principle or ALARA principle" to protect health against detrimental risks from a contaminated environment, e.g. contaminated with natural and anthropogenic pollutants. This is a guard or protective shield also against possible not yet sufficiently understood environmental-toxicological interactions, for the benefit of the current population and protection of the basis of life of future generations.

For regulatory purposes and risk management, hygiene also uses toxicological tools and methods in "risk assessment," but more imponderability must be taken into account in the development of limit values. Environmental hygiene deals with water-, soil-, air-, and food-borne, potentially harmful influences on the living nature and human being. The environmental-hygienic evaluation has to pay attention to a much larger variation width of influencing factors than, for example, the traditional occupational toxicology. Broad variances of the life circumstances and considerable differences in the length of the exposure, the number of substances, and further factors (see Table 1) exist. Also, the prevention claim of the hygiene discipline is

Table 1 Constraints and assumptions

	Toxicology/occupational health	Hygiene and environmental medicine
Exposure substances	Some few	Many
Exposure type	Mono-media (e.g., via air)	Polymedial (water, soil, air, food, toys, etc.)
Duration	8 h/day	24 h/day
	Working life (40–45 a)	Lifelong (70–90 a)
Substances (nature, number of)	Definable, known	Inconsistent knowledge, unknown
Philosophy	Exposure reduction when a limit value is reached	Preventive exposure avoidance, even when there is no current evidence for exceedance of specified limits
Use, handling	Handling by informed persons	Unpredictable handling by uninformed users possible
Health status	Healthy; under occupational medical control	Healthy and sick, old and young; no targeted control possible
Protection	Protective clothing, ventilation, air extraction	Not possible; substitution
Monitoring surveillance	Targeted occupational surveillance and investigation, targeted measurement (e.g., human biomonitoring)	Incidental findings (e.g., population-based human biomonitoring)
Substance combinations (combined effects)	Few	Many

more fare-reaching and has recently been integrated in many regulation philosophies, including occupational medicine.

The derivation of toxicologically reasonable limit values, for example, OEL values, is always dependent on the state of scientific knowledge at the time point of implementation. Thus, it is also hardly disputable that in some cases in the past, limit values were subject to be corrected and judged more strictly a few years later, and some substances subsequently had to be classified as carcinogen. This may be considered as just barely tolerable for occupational (40 h/week) exposures and under consideration of the employers' liability insurance for economic compensation in case of damage. But the possible and nearly unavoidable impact of scientific uncertainty appears not acceptable when the population is exposed during the whole lifetime and when considering the cumulative effects for future generations (e.g., ground pollution, refuse dumps).

With this in mind, any complex weighted environmental-hygienic limit value usually provides a wider scope of protection compared to a similar value that is

derived using a toxicological approach. These additional weighing processes will be clarified further using the historical example of setting drinking water limit values for pesticides.

Example: Pesticide Regulation in the Drinking Water

The different approaches are exemplifiable by comparing the previous regulations of pesticides in drinking water in Germany (predominately hygienic-environmental based) with that of the World Health Organization (predominately toxicological based). Although both aiming at apparently nearly the same objective of protection and conservation, the numerical values show that different approaches were applied: use of the chemical analytical detection limit in the German regulation but toxicologically derived values in the WHO drinking water guidelines (WHO 1993, 2017; Bundesregierung 2020).

The German Hygiene Philosophy

The environmental impact of pesticides, e.g., due to (useful) agricultural activity, would reach an absolute upper limit, if such activities would inevitably lead to a (harmful) contamination of groundwater or drinking water. On the one hand, pesticides absolute lower limit is defined by the amount of active ingredients, which must reach the target organism (pest) in order to be effective. On the other hand, it should not contaminate the non-target-compartments of soil or groundwater. The compromise between harmfulness, usefulness, measurability, and preventability of pesticides leads therefore not to a value of "zero," but to 0.1 µg/l per single active substance in ground- or drinking-water. This tolerance threshold corresponds to the state of the art and makes agricultural activity equally acceptable for positive and negatively concerned persons (Dieter 2010).

For most pesticides, this drinking water limit value corresponds to hardly 1% of the lifetime innocuous dose. Thereby, it is guaranteed that damages due to combination effects, barely investigated metabolites or disinfection by-products that may be formed during chlorination of drinking water (e.g., chloroform), can be excluded with practical certainty. This also means that a temporary minor limiting value violation would rarely result in an immediate health risk. Nevertheless, countermeasures should be applied immediately in any case, considering the sometimes decades long "contamination memory" of soil and groundwater.

The Philosophy of the WHO

WHO has the task to evaluate, generate, and distribute scientifically derived health standard. The procedure is fundamentally different from that in the German regulation: departing from an health based guidance values for total exposure, e.g. ADI or

tolerable daily intake (TDI) value as a convention 10% is allocated to the drinking water path. For lipophilic pesticides such as aldrin, dichlorodiphenyltrichloroethane (DDT), lindane, and some others substances, for which it is assumable that the main transfer in humans occurs, e.g., via the food path, the allocation is only 1%. For substances which are probably carcinogenic for humans, concentration values were derived departing from a reference risk of 10^{-5} using the usual exposure parameters for drinking water.

However, it is not expected by any means that the toxicologically derived limit values will be adopted directly into laws of the various countries. In the introduction to the "Guidelines for drinking-water quality," WHO states that for the derivation of national standards, it is necessary to consider the context of the local and national environment as well as the social, economic, and cultural conditions. In spite of references to necessary cost-benefit weighing, there is also a hint, that "*every effort should be done, to achieve a drinking water quality that is as high as possible. The best protection of the drinking water consists in avoiding the pollution of the raw water."* It is also stated: *"Although the guideline values describe a drinking water that meets the claim of lifelong health protection, their derivation must not be understood in a way, as if this approved a replenishment of a given drinking water quality up to the mentioned values. On the contrary, the protection of a drinking water quality that is as good as possible, demands and earns continuous effort"* (Dieter 1993).

WHO as supranational organization could hardly be clearer than in the quoted comments in expressing its support for a "Minimization principle or ALARA-principle" which exceeds the mere compliance with strictly toxicologically derived guideline values. This even more, because WHO must always keep in mind the specific conditions, in particular the economic situation of the less developed countries.

For the national adoption of the guidance values, WHO advises to take into account the specific ecological, geological, socioeconomic, and technical prerequisites. Therefore, the comparison of the WHO guideline values with the German legal limit values shows that the environmental-hygienic demands seem to be absolutely appropriate. In particular, they fulfill the claims for a sustainable protection of the environmental resources.

Regulation of "Unknown" Substances

There are situations, such as incidents of raw-water contamination, where drinking water regulation can unexpectedly have to deal with formerly unregulated substances and a lack of toxicology data (e.g., per- and polyfluoroalkyl substances (PFAS) contamination in Germany in 2002). In such a situation, the public health authority has to check whether there is no hygienic concern present and whether the drinking water is still compliant with the requirements of the national drinking water regulation, despite the presence of the new contaminant. Such a specific approach was developed, e.g., by the German National Drinking Water Commission and published as a Recommendation of the German Federal Environmental Agency (FEA/UBA 2003).

Cross-References

- ▶ Assessment of Limit Values in Regulatory Toxicology
- ▶ Default Factors in Quantitative Risk Assessment
- ▶ Do Carcinogens Have a Threshold Dose? The Pros and Cons
- ▶ Limit Values and Guideline Values in Regulatory Toxicology
- ▶ Precaution Principle Versus Danger Prevention in Toxicology
- ▶ Toxicological Risk Assessment in Different Jurisdictions
- ▶ Uncertainty Analysis in Exposure Assessment-Relevance for Toxicological Risk Assessment

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Protected Property and Protection Level in Regulatory Toxicology

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Bernhard Liebl and Ines Liebl

Contents

Abstract

The modern determination of standards (benchmarks, threshold values, etc.) is achieved in a multistep process, beginning with the definition of the subjects of protection as well as protection goals and levels of protection, respectively. The process is not strictly divided from step to step. The assessment of data from one step often requires a feedback to the primary subjects of protection and protection goals.

Keywords

Subject of protection · Protection goal · Protection level · Quantitative risk assessment · Threshold

B. Liebl (✉)
Bavarian Health and Food Safety Agency, Oberschleissheim, Germany
e-mail: bernhard.liebl@lgl.bayern.de

I. Liebl
Bavarian State Ministry of Health and Care, Munich, Germany
e-mail: ines.liebl@stmgp.bayern.de

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Introduction

Subjects of general legal protection such as human being itself or the animated or inanimated environment are established in the constitutions of many countries worldwide.

Besides such general determinations of subjects of protection, it is equally important to define how far the protection should go. Protection goals describe the degree of intended protection and thereby the level of protection aimed at. If a protection goal is defined, this can be substantiated by quantitative risk assessment (QRA).

These general definitions will be explained in terms of toxicology in the following chapter.

Subjects of Protection

Human being itself or the animated or inanimated environment can represent subjects of protection (Fig. 1). In this context, two objects of legal protection are of significant importance:

- "Physical integrity" (physical health)
- "Conservation of natural resources" (environment: ground, water, air, fauna, and flora)

In many countries, these objects of legal protection are firmly established in the constitution. Therefore, they have to be respected even if they are not explicitly

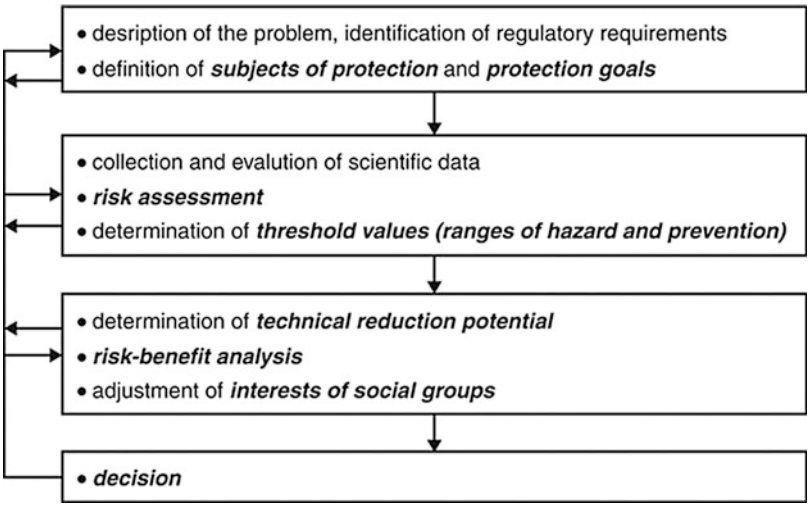

Fig. 1 Determination of environmental standards

addressed in a relevant law. Additional constitutionally protected objects, which have to be considered in this context, are "**professional freedom**" and the "common freedom of action." These basic rights are very relevant in the economic sector. They ensure the freedom to perform the profession of one's own choice, the use of manpower against payment, the possibility for businessmen to compete, and the entrepreneurial freedom of action.

Against this background, for example, in Germany, the ad hoc commission "reorganization of proceedings and structures for risk assessment and standardization in environmental health protection" (risk commission) defined three subjects of protection:

- Human life
- Diversity of species and types
- Economic power

These three subjects of protection depend on each other. They are fundamental in context of the global action program for the twenty-first century "Agenda 21" and the resulting strategy of "Sustainable Development," compiled in 1992 in Rio de Janeiro by the "Conference of the United Nations on Environment and Development," that was a basis for the United Nations Sustainable Development Goals of 2015.

When concrete measures are planned or evaluated, these three subjects of protection can come into conflict with each other. In such cases, it is recommended to distinguish between **central and peripheral areas within the subjects of protection** (Fig. 2). For human beings, the protection of health and, for nature, the protection of the natural living environment represent the central area (anthropocentric versus ecocentric protection of the environment or nature). The peripheral areas cover especially socially, culturally, and economically associated subjects which influence and determine the central areas. These subordinated, peripheral areas overlap and often cannot be precisely assigned to a distinct subject of protection. If it comes to a conflict between the central areas of the different subjects of protection, one should seek a measure which shifts the conflict into the peripheral areas, in order to protect the central areas as much as possible. In the peripheral areas, activities that carry risks become comparable and calculable. Additionally, in a concrete situation, it has to be considered that upper-level objects of legal protection – normally, life and health of human beings – are favored compared to, e.g., economic objects. Compensatory measures should be considered for more affected subjects of protection. Moreover, risks depending on external influences should receive more weight than self-dependent risks.

Protection Goals and Levels of Protection

Besides the definition of subjects of protection, it is also important to define how far the protection should go.

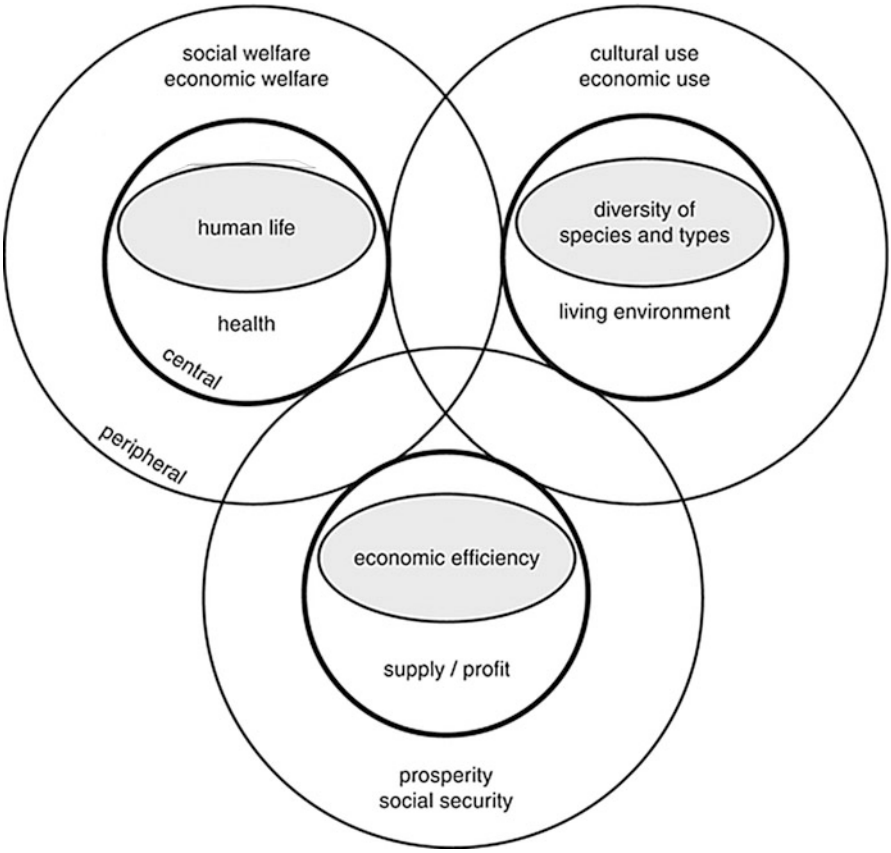

Fig. 2 Areas and subjects of protection

Protection goals describe the degree of intended protection and thereby the **level of protection** aimed at. Their definition has significant impact on the quantification of standards and the following implications. Protection goals can be classified in two ways:

- Complete protection – partial protection
- Hazard control – prevention

Complete Protection: Partial Protection

In this context, depending on the risks that are to be regulated and the subjects of protection, the following questions arise:

- Is complete protection of subjects of protection intended or are certain risks tolerable, because their complete exclusion is not possible, too expensive, or socially not accepted?

- Are entire systems (i.e., populations, ecosystems) to be protected or additionally each therein contained individual component, possibly including particularly sensitive components?

In the discussion of these questions, also constitutional criteria have to be considered, for example, suitability, requirement, and adequacy of a planned measure.

Hazard Control: Prevention

In many countries, law differs between damage, danger, prevention, and remaining (residual) risk. **Damage** means that the probability of a negative event (adverse effect) amounts to one, i.e., a negative event occurs with certainty or has occurred already. **Danger** means that damage is expected with a (inacceptable) high probability. In context of law, dangers have to be defended. The borderline separating danger from the range of prevention is determined by the level of non-tolerable risk. The borderline separating the range of prevention from a remaining (residual) risk is defined as **tolerable risk** (**traffic light principle**, Fig. 3).

Substantiation of Protection Goals: Deduction of Standards

If a protection goal is defined, this can – as far as possible and necessary – be substantiated for both protection levels, i.e., danger defense and area of prevention, respectively, by **quantitative risk assessment (QRA)**.

Generally, **danger defense** is implemented by definition of a normative **threshold value**. Threshold values generally separate the area of danger from the area of prevention. Exposures lower than the threshold values usually imply that affected objectives have no risk of damage. On the other hand, this does not imply that an exposure exceeding the threshold value automatically leads to damage.

An important source for the deduction of threshold values is toxicological data resulting from dose-effect or dose-probability estimations, respectively. In this

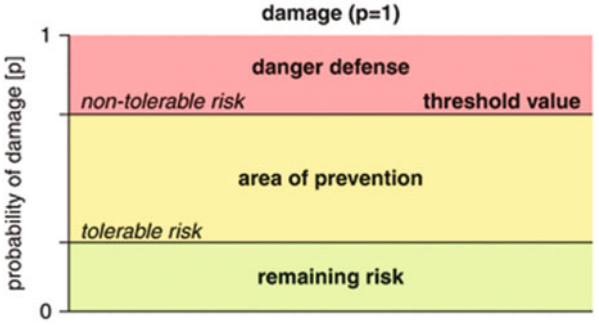

Fig. 3 Traffic light principle

context, it is important to differ between agents with dose-effect curves revealing a level beneath which no effect is observable or expected from agents for which such a level is not apparent. The last applies particularly for genotoxic agents, e.g., benzene or benzo(a)pyrene.

For **agents with a threshold of effect**, regulatory values are generally defined using the ADI concept of the WHO. Point of origin in this context is the "no observed [adverse] effect level" (NO[A]EL) or alternatively the "lowest observed [adverse] effect level" (LO[A]EL). The threshold for human beings, at which lifelong no harm for health can be expected (convention, not toxicologically evidenced), is calculated by division by a safety (respectively uncertainty) factor (normally 100).

A method used for **agents without a no observed effect level** (e.g., genotoxic agents) is, for example, the **unit risk method** of the Environmental Protection Agency (EPA). The unit risk of an agent describes the estimated additional lifelong cancer risk posed on a person exposed for 70 years with 1 μg of the agent per m^3 air. An additional lifelong cancer risk between 1:10,000 and 1:1,000,000 is discussed as acceptable. The dose corresponding to a risk of 1:1,000,000 is called "virtually safe dose."

The protection philosophy of threshold values based on quantitative risk assessment can be found, e.g., in the WHO "Air Quality Guidelines" and the "Guidelines for Drinking-Water Quality" for Europe or the "Maximum Residue Limits" of the WHO.

The **precautionary principle** implies that (environmental) exposure should be prevented or reduced far before the risk of danger occurs. This principle is particularly applied in case of a suspected risk of agents for which scientific data for (quantitative) assessment are not yet sufficient to define threshold values. This is, for instance, the case when causal correlation between an exposure and damage is likely but not (yet) proven. In these cases, the principle of exposure reduction as far as economically and socially justifiable (ALARA, "as low as reasonably achievable") or as far as technically possible (ALATA, "as low as technically achievable") can be applied. In these cases, the precautionary principle is often not related to measurable effects and refers to the principles of "sustainable development" and protection of environment for further generations.

The **protection level** aimed at the individual case (i.e., how safe is safe enough? definition of "tolerable" or "negligible" risks, respectively) and the subsequent options of action are generally defined in the course of a normative (political) process of decision-making. At best, science can contribute by describing scenarios using objective scientific data. Modern, socially accepted regulatory processes additionally require adequate information and participation of the public and transparent reproducible decision-making policies.

Cross-References

► Precaution Principle Versus Danger Prevention in Toxicology
► Risk Comparison in Toxicology

► Risk-Benefit Considerations in Toxicology
► The Regulatory Process in Toxicology
► Toxicological Risk Assessment in Different Jurisdictions

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Ethical Issues in Science: Focus on Regulatory Toxicology

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Beate Henrikus and Wolfgang Eisenmenger

Contents

Abstract

The conduction of clinical trials with medicinal products or medical devices is a mandatory requirement for the license of new, possibly more effective medicinal products/medical devices with a better risk/benefit profile.

Preclinical studies to determine pharmacokinetic parameters such as absorption, bioavailability, metabolism, half-life time, excretion mode, or toxicological properties of new medicinal products are necessary, but they are increasingly conducted on cell models in order to reduce animal experiments.

B. Henrikus (✉) · W. Eisenmenger
Ethics Committee Ludwig-Maximilians-University Munich, Munich, Germany
e-mail: Beate.Henrikus@med.uni-muenchen.de; Wolfgang.Eisenmenger@med.uni-muenchen.de

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Both animal and human experiments are now subjects to strict and clear legal and ethical regulations. The first tentative approaches to ethical regulations can be observed at the beginning of the twentieth century, which have been amended and specified many times in the meantime. At the same time, towards the end of the twentieth century, there was an increasing demand to replace animal experiments by cell models as far as possible.

Nevertheless, before starting a first-in-human (FIH) study, experiments in two different animal species are usually necessary to estimate an initial dose using NOAEL or MABEL (minimum anticipated biological effect level). Such a study on healthy volunteers is an experiment under special ethical issues, because the medical ethics principle of "nihil nocere" is violated; the healthy participants have no chance of benefit, but the risk of a potentially serious adverse effect. This contribution describes the historical background and the current ethical issues of human studies and animal testing.

Keywords

Animal replacement · Animal welfare · Ethical principles · Ethical review criteria · Studies in humans/animals

Introduction

Toxicological, pharmacological, and biomedical trials in humans or animals imply ethical issues. Due to legal requirements, these studies are subject to an ethical assessment in most countries. Although the ethical principles, the review criteria, and the legal basis have been well established and harmonized for many years, the formal ethical assessment procedure differs on several factors.

Investigators have multiple and comprehensible interests: the desire to conduct high-quality research, to complete the research quickly, to protect research participants, to obtain funding, and to advance their careers. The very nature of many ethical issues in research means that they cannot easily be defined as clearly right or wrong. The resolution of these issues relies upon the person's values and beliefs, thus requiring an independent opinion. Independent review (frequently in the form of an ethics committee) provides public accountability and minimizes potential conflicts of interest.

Much has been written about the bureaucratic downside of formal ethics review systems. On the one hand, ethics review uses up precious time and can be seen as delaying the research. On the other hand, through the continuous dialogue between researcher and the review committee, a positive reflective process is embedded throughout the experiment's life span.

In order to gain a deeper appreciation of ethical principles, it is helpful to consider the historical debate.

Historical Development

In 1833, William Beaumont, a US Army surgeon, advocated for the right to perform human experiments. In 1865 Claude Bernard, a French physiologist, argued for animal experimentation as part of the standard scientific method. Both scientists only focused on the researchers' rights. About the same time, the scientific community in medicine became aware of ambivalence in medical studies. The discrepancy between an experimental therapy, aiming at scientific interests, and an individual treatment experiment, serving patient's welfare, was published by Charles Nicolle in his views on the moral responsibility of scientists. At the end of the nineteenth century, as the experimentation on human beings and animals increased, criticism and controversy began. The public began to demand that the welfare of the patient is respected as well as the interests of researchers.

In Prussia, research regulations were introduced in 1900, following the increased governmental awareness of the lack of standards in medical research. These regulations were among the earliest and clearest pronouncements on the importance of informed consent in medical research. The reason for these research regulations was based on vaccine trials, conducted on prostitutes and abandoned children without consent.

In 1931, a directive from the German Ministry of Interior demanded that innovative or experimental therapy could only be conducted on human subjects if the person concerned (or his legal representative) had unambiguously consented after being informed in advance about the nature of the procedures and their risks.

In 1947, the "Nuremberg Code" was published. This code was based on ethical principles developed by the Nuremberg Military Tribunals during the prosecution of physician researchers, accused of conducting horrible medical experiments on prisoners of war during the Second World War. Being the first international standard for the conduct of medical research, the code was designed to protect the rights and the well-being of human subjects in medical experiments and to establish voluntary consent in research.

In 1964, the World Medical Association issued new recommendations on "Ethical Principles for Medical Research involving Human Subjects," based on the principles of the Nuremberg Code. They are known as the "Declaration of Helsinki." The Declaration has been adapted and amended several times, most recently by the 59th World Medical Association in Seoul in 2008. The declaration modified the Nuremberg Code's first principle that the voluntary consent of the human subject is essential, with the recognition of the legitimacy of proxy consent for research involving children and persons with cognitive impairment. The Declaration of Helsinki states: "The research protocol must be submitted for consideration, comment, guidance and approval to a research ethics committee before the study begins. This committee must be independent of the researcher, the sponsor and any other undue influence. It must take into consideration the laws and regulations of the country or countries in which the research is to be performed as well as applicable

international norms and standards but these must not be allowed to reduce or eliminate any of the protections for research subjects set forth in this Declaration."

In 1979, the Belmont Report was published. It summarizes the basic ethical principles developed by the United States Department of Health, Education, and Welfare, due to the problems arising from the Tuskegee Syphilis Study, an experiment in poor, rural black men. In this study the researchers knowingly failed to treat patients appropriately in order to study the natural progression of untreated syphilis.

In 1997 the International Conference on Harmonisation (ICH) published international ethical guidelines on Good Clinical Practice (GCP). These guidelines seek to harmonize clinical studies worldwide and to ensure that the data generated from studies are valid.

In 2004 the European Union implemented the principles of Good Clinical Practice. They have been laid down in the EU Directive 2001/20/EC. This Directive is currently law in all EU Member States but is to be replaced by EU Regulation 536/14 at end of 2021. The aim of the new regulation is to improve global harmonization and reduce bureaucracy. In the USA, they appear in FDA Federal Regulations Title 21, Subchapter A, Part 56.

In 2006, the disastrous results of the first application of the monoclonal CD 28 human antibody TGN1412 in healthy volunteers, a so-called first-in-human (FIH) study, raised many serious medical and ethical issues. The applied humanized monoclonal antibody acted in different fashion in humans as compared to the toxicological tests in laboratory animals. Nothing in the preclinical and toxicological tests predicted the overwhelming systemic reaction to the antibody; no previous animal tests demonstrated the toxicological response seen in humans. The severe adverse reactions occurred due to adverse immune-mediated drug reactions (such as cytokine storm, autoimmunity, and immunosuppression). As a consequence, a guidance for first-in-human studies was enacted by the European Medicines Agency (EMA) in 2007. Special care has to be paid to the novel mechanism of action (extent, amplification, duration, reversibility of the effect), the nature of the target, the relevance of animal species and models (questionable relevance implies an additional risk), the estimation of the first dose in human (when the methods of calculation (e.g., NOAEL, MABEL) give different estimations of the first dose in man, the lowest value should be used), the sequence and the interval between dosing of subjects within the same cohort, the dose escalation increments, the transition to next dosing cohort, the stopping rules, responsibilities for making decisions, monitoring, and communication of adverse events/reactions.

In 2017 the European Medicines Agency (EMA) has revised this guidance to further help researchers identify and mitigate risks for trial participants.

The differences in target affinity, mechanism of action, and immunogenicity between established toxicological models (i.e., NHP) and the human immune system, the functional potency of humanized monoclonal antibodies to modulate the target, and the new toxicology of these complex protein products (i.e., high target specificity, lack of metabolite toxicity) require the development and validation of new toxicological models.

Financial aspects must also be considered: the compensation of volunteers for the assumption of risk and the fair compensation of trial participants in case of injury.

Ethical Principles

EU Directive, FDA Regulations, as well as the Declaration of Helsinki require an ethical review on a legal basis. Furthermore, they introduce legal obligations and specifications for the scope of the ethical assessment. Ethics committees have to guarantee that investigators act in compliance with fundamental ethical principles. These principles are as follows.

Respect and Protection

A fundamental principle is respect and protection of the individual. This includes the well-being of research participants, their right for self-determination, protecting privacy by assuring confidentiality of personal information, and respecting anonymity. The privacy of research participants and the confidentiality of their personal information have to be protected to minimize the impact of the study on their social integrity.

Informed Consent and Transparency

A further principle is the right of a participant to make informed decisions, regarding participation in medical studies, both initially and during the course of the medical study. Voluntary consent must be guaranteed. No competent individual may be enrolled in a clinical trial unless he or she freely agrees. A research participant also has the right to withdraw consent at any time for any reason, without affecting their subsequent care. The participant must also be informed of all potential trial risks and burdens and of any newly discovered risks or benefits during the course of a clinical trial. Participants shall be informed of the results of the medical study.

Favorable Risk-Benefit Ratio

It is based on the principles of "non-maleficence" and "beneficence." Every medical trial has some degree of potential risk and benefit; therefore, investigators have to insure that risks to study participants are minimized. Consequently, a careful assessment of the possible risks and benefits must be carried out for the trial participants. Medical studies involving human subjects may only be conducted if the importance of the objective outweighs the inherent risks and burdens to the study subjects. There has to be a reasonable likelihood of benefit to the population studied. Experimental studies should always be compared to the best methods, but under certain

circumstances, a placebo or no-treatment group may be utilized. Special attention has to be paid to patients involved in placebo arms. The subsequent treatment of the research subjects after the end of the study is part of the ethical assessment. This includes the assurance that they will have access to the best-proven medical procedures. Investigations that are contrary to morals and conventions are ethically not acceptable. An example for such an experiment is a trial in humans for detecting the threshold of injuring effects of pesticides or herbicides. Such studies provoke only harms but no benefit for the individual.

Fair Participant Selection

This principle is based on the principle of justice. To be ethical, the selection of participants must be fair. Investigators need to ensure that:

On the one hand, stigmatized and vulnerable individuals are not targeted for risky medical experiments. Medical studies involving a vulnerable population are only justified if there is a reasonable likelihood that the population benefits from the result of the research. When a study participant is incompetent, physically or mentally incapable of giving consent, or is a minor, the investigator needs the consent of a legal representative or proxy acting in the subject's best interest. On the other hand, not only rich and socially powerful individuals should be favored for potentially beneficial research. This demand is especially relevant for nations form the so-called Third World or nations without public health insurance coverage.

Scientific Validity

To be ethical, clinical research must be conducted in a methodologically rigorous manner and must be of scientific value. Bad science is bad ethics because it does not emerge better medical knowledge but may provoke additional risks and harms for study participants. Furthermore, bad science may generate incorrect and nonvalid data which may entail harmful, risky, or ineffective treatments. The allocation of safe and effective drugs is a mandatory ethical and legal requirement.

Ethical Review Criteria

The primary task of an ethical assessment by ethics committees is the review of research proposals and their supporting documents. Therefore, special attention is paid to the informed consent process, documentation and the suitability and feasibility of the protocol. Ethical reviews need to take into account previous scientific reviews and the requirements of applicable laws and regulations. The ethical review is focused on – but not exclusively limited to – the following issues:

- Are the risks acceptable?
- Are there any precautionary measures to minimize the risks?
- What are the potential hazards and how they are handled? An example is the use of magnetic resonance tomography (MRT) as a diagnostic tool instead of a CT in order to eliminate radiation burden.
- What is the scientific validity of the proposal – will it achieve its stated objectives?
- Is the methodology appropriate to the study?
- Are the drug and its dose adequate? Is the dose used to examine the efficacy the same as used for the safety research?
- Is the sample size adequate?
- Is the use of placebo in the control arm justified? If there is a best care regime available as control that is well recognized and commonly applied, a placebo arm is ethically not justified.
- What are the criteria for withdrawing a research participant prematurely from the research or for suspending or terminating the research as a whole? Has the welfare of the participants been protected? This includes physical and emotional welfare, discomfort, and distress. The impact of the study on the participants must be anticipated.
- What are the characteristics of the population from which the research participants will be drawn? This includes gender, age, literacy, culture, economic status, and ethnicity.
- Are adequate provisions made for monitoring and auditing the conduct of the research, including the constitution of a data safety monitoring board (DSMB)?
- In which manner will the results of the research be reported and published?
- Are the conditions of insurance (insurance coverage) adequate?
- Are provisions for data protection according the corresponding law?
- Are the study sites suitable and the staffs adequately trained?
- Have human rights been respected? Was the consent obtained voluntarily? Any coercion invalidates the consent made. Is the informed consent form understandable to the potential participants, in particular, if vulnerable groups, such as children or partly incompetent patients, are involved? Is the research participant adequately informed about the nature, significance, risks, and implications of the medical study, as well as about his or her right to withdraw from the experiment at any time without affecting his or her subsequent care? A generally comprehensible information sheet is to be handed out to him. Furthermore, the person concerned is to be given the opportunity to have a counselling session with an investigator about the other conditions surrounding the conduct of the medical study. Is the right of privacy respected? The consent must refer particularly to the collection and processing of health-related data. The participant should know which data will be collected and who will have access to them.

Types of Experiments

The spectrum of experiments with ethical implications is divided into different types. One of the main types is the clinical study on drugs or medical devices in humans. This type of study is well regulated and harmonized at the EU level and the USA, respectively. ICH Topic E 6 Guideline for Good Clinical Practice Note for Guidance presents detailed written instructions to achieve uniformity of the performance of specific drugs. The methodology must be clearly described, and copies of the patient's information and consent will be required.

Another main type is the epidemiological study. It seeks to detect the incidence or the prevalence of diseases (i.e., epidemiological studies led to the discoveries of the relationship between smoking and cancer and to the identification of heart disease risk factors). Population studies demonstrated the mechanism of the transmission of AIDS and other infectious diseases and also showed how these diseases can be prevented. It also includes studies of a new medical procedure in the context of diagnosis or radiotherapy, surgery, transplantation, psychotherapy, or studies in complementary or alternative medicine as well as research experiments performed to determine how health care is delivered or might be improved or to examine personal or social behavior, opinions, or attitudes. For this type of study, a set of recommendations is available, the so-called good epidemiologic praxis (GEP). These recommendations seek to standardize epidemiological studies and include ethical aspects, research questions, study protocol, biological sample banks, quality assurance, data management and documentation, analysis, data protection, contractual conditions/frameworks, interpretation, communication, and public health. In contrast to the GCP, the GEP are only recommendations without legal basis.

Another form of studies is the category "biomedical studies" which includes many subtypes. It includes studies on human materials (i.e., blood, tissue, urine) or on human data such as questionnaire-based projects. This form of study has not yet been standardized or been regulated at the EU level. A further main type is the animal study.

Animal Welfare

In the middle of the nineteenth century, animal experiments were established as part of the standard scientific method. They include pure research such as genetics, developmental biology, behavioral studies, as well as applied research such as biomedical research, xenotransplantation, drug testing, and toxicology tests, including cosmetics testing.

One of the first opposition to the use of animals in medical research arose in the USA and resulted in the passing of the Animal Welfare Act (AWA) in 1966. In the meantime, the act has been amended several times (most recently in 2013). It regulates the treatment of animals in research. Other laws, policies, and guidelines

may include additional species coverage or specifications for animal care and use, but all refer to the AWA as the minimally acceptable standard for animal treatment and care.

Early objections to animal testing came from the belief that animals were so different to humans that results from animals could not be reliably applied to humans (i.e., contergan). There were also objections on an ethical basis, contending that the benefit to humans did not justify the harm to animals.

In 1985, a set of ethical principles known as the 3Rs, Replacement, Reduction, and Refinement, were outlined into 11 principles by the Council of International Organizations for Medical Sciences (CIOMS) and have become the international standard governing animal experimentation. In the European Union, the Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes was adopted in 1986. In November 2010, "Directive 2010/63/EU on the protection of animals used for scientific purposes," which updates and replaces the 1986 Directive 86/609/EEC, was finalized and came into force. Full implementation of the new EU directive starts on January 1, 2013.

In the last decades, there was an enormous success in the replacement of animals. Meanwhile nonanimal test methods become more and more numerous and have been formally validated and accepted by most countries as replacements for an existing animal test. A variety of cell-based tests and tissue models can be used to assess the safety of drugs. Examples include models for tumor biology with a multifunctional microfluidic-based approach as well as sophisticated in vitro, genomic, and computer-modelling techniques of cell and tissue culture; healthy or cancerous or otherwise morbid human tissue (in vitro); and induced pluripotent stem cell lines, respectively, prevalent human diseases like diabetes, cancer, heart failure, or rare diseases like cystic fibrosis and muscular dystrophy. In vitro genetic research has isolated specific markers, genes, and proteins associated with Alzheimer's disease, Parkinson's disease, muscular dystrophy, schizophrenia, and other inherited diseases. A three-dimensional model of breast cancer has been developed that will allow investigators to study the earliest stages of breast cancer and test potential treatments. Rather than studying cancer in rodents, this model, which uses both healthy and cancerous human tissue, effectively allows the study of cancer as it develops in humans. An embryonic stem cell test, using mouse-derived cells to assess potential toxicity to developing embryos, has been validated as a partial replacement for birth-defect testing in rats and rabbits. The 3T3 Neutral Red Uptake Phototoxicity Test uses cells grown in culture to assess the potential for sunlight-induced ("photo") irritation to the skin. Human skin model tests are in use, including the validated EpiDerm™ test, which has been accepted almost universally as a total replacement for skin corrosion studies in rabbits. The use of human skin leftover from surgical procedures or donated cadavers can be used to measure the rate at which a chemical is able to penetrate the skin. Furthermore there exist human lung cell model in order to test the health effects of inhaled drugs.

Another example for a well-regulated animal welfare is the system in the UK (Animals (Scientific Procedures) Act). It requires three levels of regulation:

- A project approval for the scientific substance of the project, which details the numbers and types of animals to be used, the experiments to be performed, and the purpose of them. The experiment can be performed on an animal if it can be successfully argued that it is scientifically justified and there are good reasons to cause an animal harm.
- An approval of the institution (it ensures that the institution has adequate facilities and staff).
- A personal approval for each scientist or technician who conducts any procedure. The clarification on responsibilities needs to be addressed for staff members who carry out research on animals as well as implementation of good animal welfare practices to ensure compatibility with scientific needs. In deciding whether to grant an approval, the regulatory agency has to refer to "the likely adverse effects on the animals concerned against the benefit likely to accrue as a result of the program to be specified in the license." An approval should not be granted if there exists a "reasonably practicable method not entailing the use of protected animals." The experiments must use "the minimum number of animals, involve animals with the lowest degree of neurophysiological sensitivity, cause the least pain, suffering, distress, or lasting harm and [be the] most likely to produce satisfactory results." All three licenses must be obtained before starting the animal experiment. Animal experiments can be performed based on a review and approval of the institutional official.

All regulations contain provisions to ensure that animals used in research receive a certain standard of care and treatment. Animal care and use in research are largely controlled by Institutional Animal Care and Use Committees. Most governments aim to control the number of times individual animals may be used, the overall numbers used, and the degree of pain that may be inflicted. Furthermore, there exist numerous standard operating procedures (SOPs) for animal care. Animal care duties include – but are not exclusively limited to – housing (i.e., well-controlled airflow, room temperature), daily health checks (observation for sign of illness or injury, pathogen control, general medical surveillance procedures), body weight measurements, feed, and transport (a minimum acclimation period of 72 h, physical separation of animals accomplished by housing different species in separate rooms, the extent of the quarantine period).

Although the regulations that apply to animals in experiments vary across species (i.e., stronger rules for vertebrates) and around the world, the spirit of the regulations is always the same: animal welfare!

Cross-References

► Aims and Mission of Regulatory Toxicology
► Green and Sustainable Chemistry as Regulatory Levers
► Risk Minimization in Drug Development: Regulatory Aspects

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Part V

Risk Assessment and Evaluation

Teaching Chemical Safety

49

Stefan Rakete and Dennis Nowak

Contents

Abstract

Chemical safety at work is the responsibility of every employer. He must ensure that every employee who works or has contact with hazardous chemicals is able to identify the risks posed by the chemical and is able to take measures that allow him to work safely. An important part of chemical safety is risk management. Furthermore, the correct identification, labeling, and handling of hazardous chemicals contribute to their safe use. It is imperative to teach chemical safety to every employee who works or comes in contact with hazardous chemicals and provide him with all the required information that ensures an appropriate and safe working environment.

S. Rakete (✉) · D. Nowak
Institute and Clinic for Occupational, Social and Environmental Medicine, LMU University Hospital Munich, Munich, Germany
e-mail: Stefan.Rakete@med.uni-muenchen.de; dennis.nowak@med.uni-muenchen.de

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Keywords

Chemical safety · Risk management · Hazard identification · Chemical handling · Workplace safety

Introduction

The use of chemicals by humans goes back several thousand years and today, almost everybody works with or has contact to chemicals. Thousands of novel chemicals emerge every year and are used for numerous applications, for example, pharmaceuticals, consumer goods, food products, and many others. To date, there are more than 100,000 chemicals listed in the EC inventory of the European Chemicals Agency (ECHA, EC Inventory 2020). This includes nontoxic chemicals such as sodium chloride, but also hazardous chemicals as sodium cyanide or mercury. The physicochemical properties among chemicals differ widely. It is the purpose of toxicology to determine the potential hazards of each chemical substance. In general, the safety of a chemical depends on its reactivity, concentration, the route of exposure, and use.

If chemicals are known to be harmful to humans, animals or the environment, they must be handled with care and precaution to reduce both the probability and consequences of accidents to a minimum. It is necessary that students, occupational workers, and household users who handle, store, transport, and dispose chemicals are aware of potential hazards and trained for the safe use of chemicals. The safety of chemicals depends on multiple factors: knowledge, judicious use, safe practices, appropriate methods of engineering controls, proper use of personal protective equipment, use of minimum quantities of materials, and substitution of a less hazardous chemical substance when possible.

Chemical Safety at Work

Millions of people around the globe have contact with chemicals at their workplace almost every day. This could be at a small research laboratory or an industrial manufacturer for chemicals. Typical hazards in laboratories and chemistry workplaces are

- Fire and explosion hazards resulting from flammable solids, liquids, and gases
- Risk of solids, liquids, or gases causing damage to health
- Risk of unknown, violent or reactions out of control
- Hazards affecting the eyes and skin because of caustic and irritant chemicals

It is a top priority to reduce dangers posed by hazardous chemicals to a minimum. To achieve this, general guidelines for chemical safety at work, regardless of the size

of the company, the kind and amount of chemicals used, can be followed. Simply speaking, three questions should be asked before working with any chemical or substance.

1. What can happen?
2. How likely will it happen?
3. What are the consequences if it happens?

The answers to those questions will help to minimize hazards by chemicals and are known as risk management. Doing this, we must identify hazards (e.g., a flammable liquid), their opportunity (chance of the formation of an inflammable atmosphere), and harm (explosion, fire). By weighing the evidence on the hazard, opportunity, and harm, we can choose appropriate safety measures (ventilation, absence of ignition sources) to protect us from the hazard.

Risk Management

One part of risk management is risk assessment, which should be carried out before handling hazardous chemicals. The risk assessment includes the identification of hazardous chemicals in the workplace, the activities involving those chemicals, and the possible ways in which those chemicals could cause harm to humans. The purpose of a suitable and sufficient risk assessment is the determination of measures to eliminate or reduce the risks and exposure originating from these chemicals as much as it is reasonably practicable.

The risk assessment should consider the following parameters:

- The hazardous properties of the chemical (e.g., the information on the safety data sheet)
- The specific circumstances of the work with hazardous chemicals
- The amount of the chemicals involved
- The precautions for safe handling, storage, transport, and disposal of hazardous chemicals and any waste materials
- The likelihood and duration of an explosive atmosphere, the types of ignition sources, and their likelihood (including electrostatic discharges)
- The severity of anticipated effects of any fire or explosion

The purpose of a risk assessment is the determination of measures to eliminate or reduce the risks and exposure originating from these chemicals as much as it is reasonably practicable. Risk management typically includes strategies that avoid the risk and reduce the probability or the negative effect of the risk. One strategy could be the application of the so-called **STOP** principle (Table 1). **STOP** stands for **S**ubstitution, **T**echnical and **O**rganizational measures and **P**ersonal protective equipment. The **STOP** principles should be followed from top to bottom.

Table 1 Risk management according to the **STOP** (**S**ubstitution, **T**echnical and **O**rganizational measures and **P**ersonal protective equipment) principle

Principle	Background	Example
Substitution	Can a hazardous chemical be substituted with less hazardous or harmless chemical?	Substitution of benzene with toluene
Technical measures	If a hazardous chemical cannot be substituted, which technical measures can reduce or eliminate the potential exposure or hazard?	Flammable liquids should be handled in the absence of ignition sources and in a well-vented area (e.g., fume hood)
Organizational measures	If technical measures cannot be applied, can the work be organized in a way that reduces or eliminates the potential exposure or hazard?	Reducing the amount of flammable liquids handled at the same time to prevent the formation of an explosive atmosphere
Personal protective equipment	How can the individual hazard risk be reduced to a minimum?	The use of appropriate protective gloves during the handling of hazardous chemicals

Substance Information and Labeling

This section provides information for the identification of health risks posed by a chemical. It reviews how chemicals are classified and where helpful information can be found. In general, all laboratory containers with chemicals should be clearly labeled and identifiable.

Globally Harmonized System of Classification, Labeling, and Packaging of Chemicals (GHS)

The United Nations' Globally Harmonized System of Classification, Labeling, and Packaging of Chemicals (GHS) is a globally uniform system for the classification of chemicals and their labeling on packaging and in safety data sheets (GHS Implementation 2020). The EU implemented the United Nations' GHS into EU law as the CLP (Classification, Labeling, and Packaging) Regulation, the US as the Occupational Safety and Health Administration (OSHA) standards. To date, most major countries adopted the GHS. The GHS ensures that a harmful chemical or product can be identified and is marked with a hazard pictogram, a signal word (e.g. danger) if necessary, hazard and precautionary statements, and information about the supplier.

Hazard Pictograms

Unique hazard pictograms (Table 2) enable an intuitive and immediate recognition of dangers posed from a chemical. Specific pictograms warn of physical dangers (e.g., explosive, flammable), health hazards (e.g., toxic, health hazard), and environmental hazards. Additionally, there are specific pictograms for the transportation of hazardous chemicals.

Table 2 GHS hazard pictograms used for rapid hazard identification

Symbol	Code	Hazard category
	GHS01	Instable explosive substances, mixtures, and products with explosives, self-reactive substances and mixtures, organic peroxides
	GHS02	Flammable, self-heating, self-reactive, pyrophoric, water-reactive, organic peroxides
	GHS03	Oxidizing
	GHS04	Gases under pressure, compressed, liquefied, frozen, dissolved gases
	GHS05	Corrosive to metals, corrosive to skin, severe eye damage
	GHS06	Acute toxicity
	GHS07	Caution (in combination with other hazard pictograms)
	GHS08	Multiple health hazards
	GHS09	Harmful to the environment

Hazard (H) and Precautionary (P) Statements

Within the GHS, H/P statements replaced the former Risk and Safety statements (R/S statements). H statements describe the type and the severity of danger posed by a hazardous chemical or mixture. The P statements describe recommended measures to limit or avoid harmful effects due to exposure to a hazardous chemical or mixture during use or disposal.

H statements can be grouped into physical hazards (H200 to H290), health hazards (H300 to H373), and environmental hazards (H400 to 420). Additionally, there are country specific H statements for the EU (EUH001 to EUH401) and Australia (AUH001 to AUH071). Physical hazards can be explosions, fires, and corrosion of metals. Health hazards describe the health effect (harmful, toxic, fatal, sensitizing, and carcinogenic), target organ (eye, skin, and lung), and the route of exposure (ingestion, skin contact, and inhalation) of a chemical. Environmental hazards describe effects on aquatic life and public health.

P statements can be grouped into general precautionary statements (P101 to P103), prevention statements (P201 to P284), response precautionary statements (P301 to P391), storage precautionary statements (P401 to P422), and disposal precautionary statements (P501). General P statements request to have the container at hand if medical advice is needed, to keep chemicals away from children and to read the label before use. Prevention P statements serve as a guideline to prevent adverse effects, for example, by only using a chemical in a well-ventilated area or outdoors. Response P statements describe specific instructions after exposure or other chemical-related incidents such as a fire, for example, to rinse the skin after contact with hazardous chemical. Storage P statements include requirements for the proper storage of chemicals, for example, to store a chemical in a dry place or in a closed container.

In many cases, multiple H and P statements apply. For instance, the combination of H302 (Harmful if swallowed) and H332 (Harmful if inhaled) results in H302+332 (Harmful if swallowed or if inhaled), the combination P235 (Keep cool) and P410 (Protect from sunlight) results in P235+410 (Keep cool, protect from sunlight).

Safety Data Sheets (SDS) and Operating Instructions

The SDS, sometimes also referred to as material safety data sheet (MSDS), or product safety data sheet (PSDS), lists information related to occupational safety and health. For every commercially available chemical or mixture, a SDS must be provided by the manufacturer. They assist professional users with important data and handling recommendations when dealing with chemicals and mixtures in order to be able to take necessary measures for health protection, occupational safety and environmental protection. It is mandatory to consult the SDS before handling a hazardous chemical or mixture. A SDS must be made available as long as the chemical is used or stored in the laboratory or workplace. Safety data sheets consist of multiple sections. Among other information, they inform about possible hazards and include hazard pictograms as well as H and P statements (section 2), describe first aid measures (section 4), and recommend actions for firefighting and after

unintended release (sections 5 and 6). Furthermore, they contain information about handling and storage (section 7), toxicological information (section 11 and 12) as well as statements about transportation of hazardous chemicals.

Based on the SDS, operating instructions are generated for any chemical or biological agent and their mixtures based on their specific use in laboratories or work places. In contrast to a manual, operating instructions solely indicate hazards and protective measures. They contain information about the specific use, hazards to humans and the environment, safety precautions, and measures. SDS as well as operating instructions must be provided to everyone working with hazardous chemicals.

Handling

Before working with hazardous chemicals, it should be evaluated if and how a safe handling can be ensured. This includes technical protective measures at the workplace, appropriate personal protective equipment, and emergency procedures in case of accidents.

Technical Protective Measures: Hazards in laboratories and other chemical workplaces can be prevented significantly by an appropriately designed and equipped workplace. This includes structural measures, the building infrastructure, laboratory and safety equipment and the properties of devices, apparatus, and other appliances. An unsuitable workplace situation can increase the risk of accidents as well as if the accidental release of hazardous materials. Ideally, laboratories should have a documentation area where written work is physically separated from chemical work. The workplace must be equipped with an adequate number of escape routes, exits, and emergency showers that reflects the local conditions as well as the materials and work methods used. Floors, their coverings, and cable conduits must be watertight to prevent chemicals that have been spilled seeping away unnoticed before they can be removed. The same applies to workbenches. Additionally, they should be resistant to chemical and thermal stress.

Laboratories and workplaces must be equipped with technical capacities that ensure adequate ventilation at all times. Fume hoods should be preferably used when working with hazardous chemicals. They are typically enclosing five sides of a work area and commonly located at a standing work height. Fume hoods prevent that (a) gases, vapors, and dusts in hazardous concentrations or quantities enter the laboratory from inside the fume hood, (b) the formation of explosive atmospheres inside the fume hood, and (c) the splashing hazardous materials or flying broken glass. Fume hoods must be made of materials that withstand mechanical, chemical, and thermal stressors and be equipped with a sash that should be closed when the fume hood is not in use.

Personal Protective Equipment: The use of personal protective equipment (PPE) depends on the work procedures and chemicals used. The selection of PPE should be determined before starting to work with chemicals. The PPE can consist of sturdy footwear, a lab coat, and protective glasses. The lab coat should be made of a fire-

resistant material. The use of additional PPE, such as protective gloves, should be according to the properties of the used chemicals. For example, depending on the physicochemical properties of an organic solvent, protective gloves specified for the handling of the respective solvent should be used. Otherwise, the safe use cannot be guaranteed.

Emergency Procedures – Everyone working with hazardous chemicals should become familiar with the local emergency procedures. This includes emergency escape routes, the location of emergency showers, and fire extinguishers and related precautions. Escape and rescue plans with clear instructions should be placed at strategic, visible locations and followed in case of an emergency. The supervisors are responsible for ensuring that the number of people working is known and be consulted in the event of an emergency.

Storage

All hazardous chemicals should be kept at appropriate storage locations with the correct labeling. A chemical inventory management system, which as a minimum includes an inventory list, helps to monitor chemicals on a regular basis. Safety Data Sheets (SDS) must be readily available for all hazardous chemicals stored, and these should be referred to for advice on storage, accidental release measures, and incompatibilities. Storage spaces for hazardous chemicals must be connected to an exhaust air. The interior of these storage spaces must be lined with material that does not burn easily. In the case of hazardous liquids, an adequately dimensioned collection pan must be located below the containers. Flammable liquids should be stored in solvent cabinets, compressed gases in gas cabinets. Hazardous chemicals used and stored outside of designated storage locations, for example, at a workbench, should be reduced to the amounts needed for the current work or experiment.

Incompatible chemicals should be segregated to avoid accidental adverse reactions that may occur due to container breakage in the storage areas. For the segregation of chemicals, all hazardous properties have to be identified (see above). Quantities of chemicals should be kept to a minimum and any bulk quantities of individual chemicals may be stored at external facilities (e.g., central chemical storage). The following table provides a general segregation scheme for the storage of selected chemical groups (Table 3).

Disposal

The generation of hazardous waste should be avoided as much as possible. This should be considered when planning experiments and work with hazardous chemicals. All hazardous waste should be kept at appropriate waste storage locations. Storage spaces for hazardous waste must be connected to an exhaust air. The interior of these waste storage spaces must be lined with material that

Table 3 Segregation scheme for the storage of selected chemical groups (green: compatible; red: incompatible, must be segregated).

	Flammable liquids	Oxidizers	Organic acids	Inorganic acids	Bases
Flammable liquids					
Oxidizers					
Organic acids					
Inorganic acids					
Bases					

does not burn easily. In the case of hazardous liquid waste, an adequately dimensioned collection pan must be located below the containers. Similarly, to the storage of chemicals, hazardous waste should be segregated in compatible groups and clearly labeled according to its content. The disposal of hazardous chemicals should be according to the guidelines of the company as well as federal regulations. Waste storage places should be checked for leakage or spills on a regular basis. The waste containers should be picked up or disposed by internal or external waste management personnel as soon as local storage locations reach their official capacities.

Implementing Chemical Safety at Work

Country-specific laws and regulations require employers and employees to follow chemical safety rules. It is the employer's duty to determine whether the employees carry out activities with hazardous chemicals or whether hazardous chemicals can be released during workplace activities. If the employer does not have the necessary knowledge to carry out the risk assessment himself, he is obliged to seek expert advice. For this, the employer can appoint a knowledgeable person as a representative for hazardous substances. The central task of the hazardous substances officer is to advise the employer, but also employees, on all questions relating to the handling of hazardous substances and to carry out a risk

assessment. In order to be able to perform the tasks as a hazardous substances officer, he must have the appropriate knowledge. This includes:

- Regulations of chemicals (GHS)
- Safe handling of hazardous chemicals
- Storage and internal transport of hazardous chemicals
- Labeling of hazardous chemicals
- Operation instructions and safety data sheets
- Behavior and measures in the event of accidents

All employees should receive proper chemical safety training by the employer or the hazardous substances officer before working with hazardous chemicals. Chemical safety trainings should be repeated on a regular basis, for example, once a year, to refresh knowledge and provide updated information. It should include general information as described above but also workplace-specific information.

Good Laboratory Practice

Good Laboratory Practice (GLP) is a formal framework by the Organization for Economic Co-operation and Development (OECD) for the nonclinical safety testing of chemicals, pharmaceuticals, pesticides, and food additives (OECD 2020). GLP is required by law in many countries if the results of the tests will be used to assess the possible dangers for humans and the environment in the course of an approval, permission, and registration or communication process. The implementation and regulation is carried out by national authorities. In contrast to safety measures, GLP ensures the uniformity, consistency, reliability, reproducibility, quality, and integrity of the tests. It determines the organizational processes and the conditions under which laboratory tests are planned, carried out, and monitored. It also includes guidelines for recording and reporting of results.

Summary

This chapter provides basic information of how to identify risks by hazardous chemicals at the workplace. However, every process or experiment takes place under conditions that are specific for the individual workplace. Although there are general concepts for chemical safety, each potential risk posed by a hazardous chemical should be assessed by an individual risk assessment. Furthermore, each individual employer and employee should familiarize themselves with the risks associated with chemicals used in a specific process and should have access to relevant information about safe handling of chemicals. They also have to keep in mind that working with hazardous chemicals always involves some degree of risk and that a concerted effort is necessary to reduce potential exposure and dangers to human and environmental safety to a minimum.

Cross-References

- ▶ Importance of Physicochemical and Physical Properties for Toxicological Risk Assessment
- ▶ Notification of Hazardous Mixtures and Cosmetic Products for Poisons Centers in the European Union
- ▶ Risk Management in Toxicological Disasters
- ▶ Toxicological Risk Assessment

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Current Role of the Risk Concept in Regulatory Toxicology

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Rolf Hertel, Michael Schwenk, and H. Paul A. Illing

Contents

Abstract

Since people live together, they must try to answer the question whether what they do causes a risk to others. When people live in proximity to one another, society sets goals. These include avoidance of creating unnecessary risks to others, minimization of unavoidable risks, and seeking to make the residual risks as predictable as possible. However, what one person perceives as a necessary risk or an evil that should be tolerated may be considered by others as a threat. In regulatory toxicology, this phenomenon must be considered when determining courses of action.

R. Hertel
Formerly: Federal Institute for Risk Assessment, Berlin, Germany

M. Schwenk (✉)
Tübingen, Germany

Formerly: Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

H. P. A. Illing
Formerly: Centre for Occupational and Environmental Health, University of Manchester, Manchester, UK

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Introduction

Many risks are associated with human activity. When the consequences of an activity are uncertain, this activity may be beneficial or it may be harmful (cause detriments). The concepts of risk and benefit characterize the consequences of any action. Risk involves a probability statement, the likelihood or frequency of the event occurring or the effect being observed, as well as a quantitative statement, identifying the extent and type of harm (detriment).

Damage (harm, detriment) has occurred when a physical or functional impairment is recognized to be the result of an activity. Damage can be determined only on a relative scale; for the toxicologist, this is the ill-health effects (including death), either on humans or on other species. In addition to acute damage and possible harmful effects visible only after prolonged or repeated dosing or in successive generations (e.g., cancer or detrimental effects on reproduction and development of offspring), reversibility of the damage must be included in an overall assessment. An internationally accepted standard methodology for quantifying damage is not yet available, although quantification is being attempted using methods based on direct monetary values and on values associated with particular effects in terms of quality of life for those harmed.

History of the Risk Term

The concept of risk has its origins in Italy and symbolizes semantically the process of venturesome circumnavigating a cliff. If you want to capture the historical dimension of the concept of risk, you will find the first hints of a deliberative decision-making in the ancient skeptics. In Pharisaic Judaism from 500 AD on, the text of the Bible and tradition were interpreted according to the requirements of the situation. Probability was considered but without the ability to undertake calculations of probability. In Europe until the late Middle Ages, the Christian personality was so dominated by religious forces that he or she was not able to make a free decision between alternatives that would be based on his own power and conviction.

The first systematic solution to a probability problem, that of playing dice, was recorded in the correspondence between Fermat and Pascal in 1654. At the beginning of the Enlightenment, Thomasius gave a hint that decision-making requires "courage and boldness." In overcoming medieval thinking (or lack of thinking) concerning both the continuing craft skills and the explicability of natural phenomena, there was a need to see the development of technology as purposeful. Groundbreaking is the invention of the lightning rod in 1752, which put the people in a

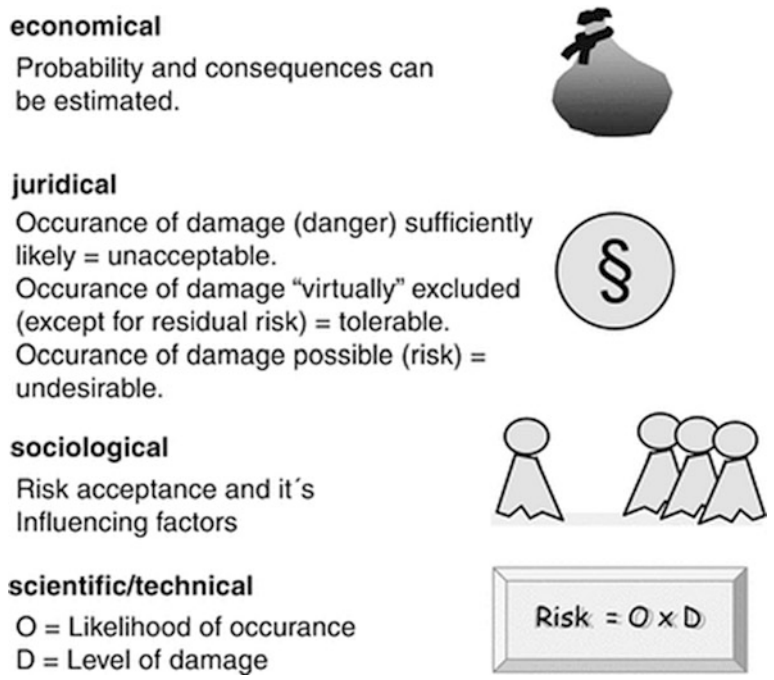

Fig. 1 Dimensions of risk

position to protect their property during a thunderstorm. The realization that the lightning strike is no longer solely an act of God but a manageable phenomenon led to the analysis of other events which were previously considered as uncontrollable. It certainly was of importance that Jesuitical casuistry was taken up as part of the general philosophy of science. Once this had happened, it was possible to develop the modern concept of risk, based on mathematical descriptions of phenomena and probability theory. Today, the term "risk" is used with varying meanings in economic, legal, sociological, and scientific/technical fields (Fig. 1).

Dimensions of the Concept of Risk

Economically speaking, decisions can be made with uncertainty concerning the risk being taken: We call it uncertainty when all the possible consequences of an action are known, but the actual outcome is uncertain. If, in addition, the likelihood of occurrence is known, we call it "risk."

The economic benefits of an activity can be optimized when one of several options for action is preferred, namely the one in which the desired sequence of actions occurs with the highest probability. The risk of being confronted with undesirable consequences of action may be low in this case but still exists. This

approach can be demonstrated using examples based on the successes (or otherwise) of different investment strategies.

Legal perspectives on the concept of risk will depend on the jurisdiction. From a German legal perspective, the concept of risk can be made clear, as it is distinct from the terms danger and residual risk. The term "danger" comes from the police law. There, the consequences of an action or activity must result with reasonable certainty in unacceptable damage. In our society, the state is obliged to avert such danger. This is the basis of laws that society has imposed on itself; the concept of danger is therefore defined by society. It also has important significance in safety and environmental legislation. When one speaks of a residual risk, absolute certainty is not given, and the non-excludable damage is accepted.

When there is a risk, a detrimental event cannot be excluded. Such a consequence is undesirable but still possible. If the damage is likely to be delayed, severe, and/or irrecoverable, the risk should be minimized by minimizing exposure (this is a statement of one form of the precautionary principle).

Sociologists and psychologists analyze how society and individuals deal with risk and with the insecurity and uncertainty of the consequence of an action. They are essentially concerned with the ways in which different groups within society perceive risks. The risk discussion is mainly concerned with risk acceptability. Often scientists are thought to deal in objective (or numerical) risk and the general public to consider risk in a subjective or judgmental manner. Depending on the perspective, the consequences of an action are considered by some sociological groups as manageable risk and by others as a threatening danger (others may feel unaffected by the risk); affected people articulate their concerns. Often, the residual risk is considered unacceptable by some or many of these groups of individuals, and they may then participate in public discussion of an issue in order to influence wider opinion.

The **scientific/technological** risk concept defines risk as the product of the extent of damage (disease/danger) and probability or frequency of the event occurring. From this simplified mathematical formula, a continuous description of all possible risk scenarios can be derived due to the variability of the factors. Here, risk is quantifiable. The Division of Toxicology of the IUPAC (International Union of Pure and Applied Chemistry) in its Glossary (IUPAC 2017) gives definition of risk: (1) the probability of adverse effects and (2) the expected severity of an adverse effect. In regulatory toxicology, the distinction between hazard (potential danger) and risk is of great practical importance.

The qualitative description of the harmful effects, imposed by a substance (inherent toxicity, hazard), is used to characterize and classify this material. As part of the IOMC (Inter-Organization Program for the Sound Management of Chemicals), the "United Nations Globally Harmonized System of the Classification and Labeling of Chemicals" (GHS) was adopted in 2002 after long discussion. GHS has the purpose to contribute to the worldwide harmonization of national communications systems on hazards posed by chemicals and thus protect people and the environment worldwide. This was based on the UN Recommendations on the

Transport of Dangerous Goods and the earlier classification and labeling systems in the EU, the USA, and Canada. Harmonized were criteria for the classification and definitions of risk potentials of substances and formulations and the elements of labeling. Most of the GHS has been formally enacted in the European Union through EU Regulation 1272/2008 (the Classification and Labeling Regulation).

To define the toxicological risk posed by the substance, its toxicity expressed as a dose–response assessment is combined with the likelihood of exposure. If an exposure is expected that would lead to an adverse effect on basis of the dose–response relationship, the risk can be quantified.

Quantification of Probability

The risk is associated with the likelihood or frequency of exposure(s) of at least a certain duration and magnitude taking place. Such a concept of probability is difficult to describe objectively, although quantification is conducted, for example, using empirical statistics. This differentiates the term "probability" from the term "suspicion." When trying to quantify, both the variability that is the actual scattering of quantifiable parameters and the uncertainty that is the uncertainty of the examiner must be considered.

Variability is the actual heterogeneity of the studied parameters; thus, it affects the accuracy of a statement. **Uncertainty**, however, can lead to false statements because it includes not only statements concerning the reliability and adequacy of a validating study at identifying and quantifying known effects but also includes allowance for possible inadequately quantified and nonidentified detrimental effects.

It is important for the further action of the toxicologists that different consequences result from variability and uncertainty. Often, uncertainties can be reduced by undertaking additional tests or involvement of additional expertise, although there is always the possibility of nonidentified effects being unknown effects appearing for the first time. Variability cannot be eliminated, however, and therefore prompts the regulatory toxicologists to adjust his protective measures, when a certain level of safety is to be maintained.

Risk Comparison

Often it is not enough for regulatory toxicologists to describe the risk posed by a substance, but it is also necessary to compare the risk with that of potential "substitutes." For this, detailed information about the **toxicological potency** of different substances is required.

In the derivation of parameters, one has to distinguish between those substances for which a threshold can be specified on the dose–response relationship and those substances for which such a threshold is considered inappropriate. One threshold measure is the acceptable daily intake (ADI). Originally, the ADI was for food

additives and then pesticides. It is the maximum level, according to present knowledge, considered to exclude a risk even when the consumer is exposed daily during his lifetime. The corresponding parameter for industrial chemicals is the "derived no effect level." These are obtained by applying standardized factors to a parameter such as the "no observed adverse effect level" or the "benchmark dose." For substances for which no threshold dose can be determined, there are several approaches. One, the unit risk and potency factor were originally introduced by the US EPA (1986). The dose descriptor T 25 can be applied to create a potency factor. T 25 is defined as the dose (in mg/kg body weight/day), which causes a tumor incidence of 25% in experimental animals after lifetime exposure. This value can be converted to the corresponding human HT 25 by being divided by an appropriate "scaling factor." This metabolically and physiologically legitimated factor is derived from the comparison of metabolic rates. The third approach is to estimate a "derived minimal effect level" using, as the starting point for extrapolation, a dose at which no excess of tumors was experimentally detectable.

Just as the concept of risk can be understood in different ways, so the perception of risk is possibly different and subjective. The broad approach, which the sociological risk discourse opens, allows for the conclusion that a comparative risk assessment and evaluation is only effective if all the discourse participants have similar basic attitudes (e.g., social background, education, interests, similar life experiences, lifestyles, and desired goals in life).

A comparative examination of the risk posed by, for example, a defined household chemical with the risk posed by a substitute can be used meaningfully if effects and exposure scenarios are directly comparable. Whether different less serious and reversible toxic effects are equivalent may need discussing. Due to methodological problems (e.g., the classic "apples versus pears" scenario as in comparing, e.g., death from smoking cigarettes vs. prize in the lottery), there are limitations when undertaking these comparisons (but see ► Chaps. 58, "Risk Comparison in Toxicology," and ► 59, "Risk-Benefit Considerations in Toxicology").

Phantom Risk

The different perceptions of risk can lead to a phenomenon that is described by the term **phantom risk**. In general, this means that different assumptions about risky cause–effect relationships are made, which may remain unprovable. Such an approach may result in an assessment that unduly increases a potential risk (exaggerated fears). Similarly, new information that is made available often leads initially to an exaggerated perception, in which the risk appears greater than it actually turns out to be later. To what extent this behavior is phylogenetically useful to sustain human life during evolution, and thus is largely unalterable, remains to be established. However, the regulatory toxicologist has to take into account this phenomenon since it directly influences the general political decision on the classification of a risk, be it unacceptable or undesirable, although it cannot be proven on a rational basis.

Dealing with the Concept of Risk

Both the definition of "risk" and the methodological processes that must be applied when dealing with questions of risk management are dependent on expert judgments. Thus, although the GHS system can provide a basis for common judgments concerning the hazard, one cannot assume that the same legal base for managing the risks will pertain internationally. In the event that no binding requirements/laws exist, all aspects of the decision finding must be presented as far as possible in a transparent way. New forms of chemistry-related global challenges will be identified. Examples are the control of atmospheric chemicals and of climate change, safe handling of toxic metals in novel electrical devices, new technologies to recycle plastic and garbage or the development of safe drugs/vaccines against newly identified infectious disease. Toxicologists (including environmental toxicologists) are accustomed to analyze and understand complex systems. Both now and in the future an understanding of risk assessment and risk management methodologies will be important when seeking sustainable development.

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- ▶ Purpose and Methods of Risk Management in Regulatory Toxicology
- ▶ Risk Comparison in Toxicology
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Importance of Physicochemical and Physical Properties for Toxicological Risk Assessment

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Hans-Joerg Burger and Michael Schwenk

Contents

H.-J. Burger
Formerly: Merck & Co., Kenilworth, NJ, USA

Morristown, NJ, USA

M. Schwenk (✉)
Tübingen, Germany

Formerly: Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

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Abstract

Physicochemical parameters are indicators for the identity and purity of a substance. Molecular size, surface charge, lipid and water solubility, vapor pressure, and other properties affect routes of human exposure and toxicokinetics. Volatility and resistance to environmental degradation are important causes of environmental distribution and persistence, and, combined with lipophilicity, they constitute a risk for bioaccumulation. This contribution describes basic physicochemical characteristics and provides examples of their relevance to toxicology.

Keywords

Solubility · Hydrophilic · Lipophilic · Volatility · Vapor · Nanoparticles · Polymer · Photoreactivity · Toxic interaction

Introduction

Each chemical has a large number of characteristic physicochemical and physical features that define its identity as a unique substance. Such features include size, charge, solubility, and many more. Physicochemical properties codetermine the routes and extent of exposure, toxicokinetics in the human organism, and (adverse) interaction with biomolecules. Moreover, physicochemical parameters are important determinants for the fate of a substance in the environment, in essence distribution and persistence. Therefore, knowledge of physicochemical and physical parameters is important in all areas of pure and applied toxicology.

Guidelines concerning physicochemical requirements and testing are available for pharmaceuticals, for example, from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH 2020), as well as national and supranational pharmacopoeias. Testing guidelines for physicochemical parameters of chemicals are part of the internationally harmonized guidelines of OECD (OECD 2020), and physicochemical features are integrated in the Hazard and Precautionary Statements of the Globally Harmonized System (GHS 2020) and in Safety Data Sheets. The appropriate interpretation of physicochemical properties, adapted to the situation or scenario, is an essential component of toxicological risk assessment and a basis of regulatory toxicology.

State of Matter

Under environmental conditions, relevant to toxicology, one commonly differentiates among three states of matter: solid, liquid, and gaseous; the transition from one state to another is dependent on pressure and temperature. Molecules in a solid phase cannot move freely due to strong intermolecular interactions. The melting point is the temperature, above which molecules can leave their fixed position, though they are still held together by intermolecular bonding forces, resulting in a liquid.

Above boiling point, the kinetic energies of molecules in a liquid exceed the intermolecular bonding forces; the molecules evaporate into the gaseous space. Some molecules tend to possess sufficient kinetic energy to evaporate even below the boiling point. The vapor pressure of a substance is a parameter that quantifies this tendency.

Information about the state of matter of a substance in question and its transition temperatures is important in toxicology, since it affects the route of exposure. The airways will preferentially be exposed to gases and vapors or dust, skin to liquids, and the gastrointestinal tract to solids and liquids. It is also of consequence for ecological chemistry and ecotoxicology, such as atmospheric transport of volatile and particulate materials and their sedimentation or the migration of a chemical in soil. Here follow some examples:

Solid Phase

Solid-phase materials may cause risks in different ways, e.g., by overdosing of pharmaceuticals or addictive drugs or ingesting toxins with contaminated food. Skin contact with solid allergenic metals such as chromium or nickel alloys may cause irritation and allergies. Solid implant materials are designed not to release substances (monomers, metals) after implantation, but minor risks remain. Inhalation of solid-phase particles like asbestos fibers, titanium oxide particles, or metal fume at the workplace is associated with lung disease. Solid plastic items may emit volatile ingredients, such as monomers or stabilizers.

Liquid Phase

Liquid phases may consist of either a single, pure component, such as water, mercury, or an organic substance or a homogenous mixture of a liquid solvent and solutes. To estimate the toxicological hazard of a liquid, it is important to know all the components that it contains.

Water represents the most abundant and important hydrophilic liquid. Its bent molecular structure together with the polar bonds between hydrogen, which has a positive partial charge, and the oxygen with negative partial charge makes water a polar molecule with a high dipole moment (1,85 Debye). This is also the origin of the characteristically strong tendency for hydrogen bonding of water molecules. Water

hydrolyzes solid organic molecules like sugars and many solid salts (e.g., sodium chloride) via electrostatic interactions and dissociates the latter into dissolved anions and cations, characterized by the dissociation constant that determines the ratio of dissociated molecules under specified conditions.

Organic liquids, such as octane, propanol, or acetone, are liquids at ambient temperature and are suitable solvents for many solid and organic substances or diluents for other liquid organic compounds. Lipophilic organic molecules typically interact with neighboring molecules via weak Van der Waals forces.

Gaseous Phase

Though the ambient air consists predominantly of the gaseous components dioxygen and dinitrogen, it also carries smaller amounts of other gaseous materials, volatile liquid molecules, liquid aerosols (fumes), and solid ultrafine particles (dust) of natural and anthropogenic origin. Synthesized toxic gases (e.g., ammonia, phosgene, chlorine, carbon monoxide) that may be released into the ambient air during a chemical reaction or from a defective gas container constitute a sometimes unnoticed risk.

Structural Aspects

Molecular size or composition by itself is only poorly associated with toxicity. One finds very toxic agents among "small organic molecules" (e.g., carbon monoxide, benzene) or large molecules (e.g., botulinum toxin protein) as well as among multimolecular ultrafine particles (e.g., asbestos).

Small Organic Molecules

The majority of industrial organic chemicals and therapeutic drugs are small organic molecules (< about 600 Dalton). Their molecular composition and positions of side chains and functional groups largely determine their individuality and their chemical name according to the rules of the International Union of Pure and Applied Chemistry (IUPAC 2020). Features such as chirality may decide about their interactions in a three-dimensional molecular surrounding, such as the binding pocket of a protein: one stereoisomer often exhibits much higher binding affinity than the other.

In the organism, most small xenobiotic molecules do not simply move according to physicochemical rules (see below). In the blood, they tend to bind to plasma proteins, notably albumin. They can be carried across cell membranes via organic cation transporters (OCT), organic anion transporters (OAT), or ABC transporters. Most small molecules are metabolized by biotransformation enzymes, which results in a change or loss of biological activity and altered physicochemical features of the products. Sometimes biotransformation involves reactive intermediates that are

genotoxic and cell toxic. Small molecules with a diameter of less than 6–8 nm fit through the renal glomerular filtration apparatus often followed by either reabsorption or secretion in the primary urine. Another elimination route, used by many conjugated metabolites, is via active transporters on the canalicular membrane from the liver cell into bile.

Organic Macromolecules

Organic polymers, whether engineered or of biological origin, are composed of repeating molecular units (monomers), arranged as linear or branched chains. The chains self-associate also via non-covalent bonds and entanglement.

Most synthetic polymers are nondigestible in the intestinal tract. Nevertheless, they pose a health concern, when residual monomers or stabilizers are released, for example, from indoor plastic materials into the surrounding air (e.g., in automobiles) or from drinking bottles into the drink or from infusion tubes into the fluids. Many plastic materials that are disposed of in the environment tend to be quite resistant to chemical decomposition and get physically torn into tiny pieces to" microplastic," which constitutes a huge environmental problem and a threat to sea life. Incineration of polyvinylchloride, or other plastic material in combination with chlorine, is associated with formation of polychlorinated dibenzodioxins (PCDD).

Biopolymers

Biopolymers are of utmost importance in toxicology, firstly, as targets of many toxic actions and, secondly, as biopharmaceuticals that must be toxicologically characterized before getting to the market. The major groups of polymeric biomolecules are proteins, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and carbohydrates.

Proteins are large molecules, based on a set of 20 different amino acids connected linearly in a genetically determined sequence (primary structure). When they are synthesized, chaperons support the normal folding of the polypeptide chains, which results in secondary structure like beta-sheet, folded tertiary structure, and spatial motives like the beta barrel. In addition, many proteins get modified with phosphate, lipid, or carbohydrate groups. These additional modifications are required to (in) activate them or direct them to a particular location within the cell. Proteins have binding sites to interact with other biomolecules.

Dietary proteins get degraded by gastrointestinal proteases, however not always completely. Some proteins are protected from digestive proteases, as in the case of botulinum neurotoxin, or of wheat gluten, the latter causing immunological intestinal disease (celiac disease) in predisposed persons. Proteins (of pollen, house mites, animal hair) may have epitopes that make them allergenic. Cyclic peptides occur in mold, some are known for toxicity (amanitin, phalloidin), and others are used as therapeutics to suppress immune reactions (cyclosporine). The bacterial AB toxins (e.g., botulinum toxin) typically consist of two proteins, one anchoring to surface

components on the target cell and inducing endocytosis into the cell where the other component then exhibits destructive enzymatic activity.

Double-stranded deoxyribonucleic acid (DNA) in the cell nucleus consists of two corresponding DNA strands each composed of a set of four DNA-specific nucleotides; the two chains are stabilized by weak chemical forces between the corresponding base pairs. The very long DNA strand is organized in nucleosomes, where DNA is wound around histone proteins. DNA is the target of genotoxicity. When DNA is ingested as a normal nutritional component, the strands are split into nucleotides that may be absorbed, and it has no role as toxic agent (except in the form of DNA viruses).

Human ribonucleic acids (RNAs) are single-stranded ribonucleotide polymers, each characterized by the sequence of its nucleotides, length, and types of folding. RNAs occur as carriers (m-RNA) and decoders (t-RNA) of genetic information from DNA to protein, as structural component of ribosomes, and as regulators of protein synthesis (mi-RNA). The development of RNA-based therapies and vaccines will afford a toxicological characterization of the RNA in vitro and in vivo, an emerging new field for toxicology.

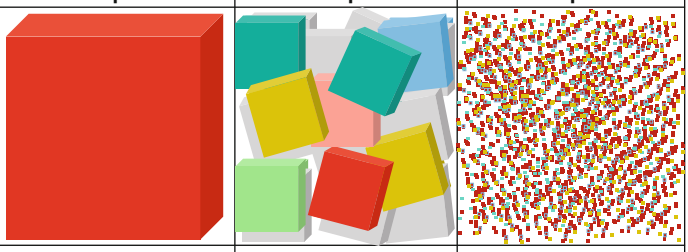
Carbohydrate polymers occur in plant-derived nutrients as digestible starch, partly digestible oligosaccharides, and nondigestible cellulose. Glycogen is a branched biopolymer consisting of glucose units, predominantly stored in liver and muscle cells as rapidly available glucose (energy) source.

Particles and Nanoparticles

Fine particles in the air appear as dust. In an occupational situation, dust may be composed primarily of a single material, such as asbestos or silicon dioxide. But in the broader environment, fine particles originate from many different sources, such as volcanic activity, incineration processes, mining, ground erosion, plant pollen, and textiles. In cities, emissions from vehicles, industrial processes, heating systems, and tire abrasions tend to be primary sources, and on New Year's Eve, the hazy air is due to metal-loaded particles released by fireworks. Taken together, atmospheric particles are a mixture of fine particle species of varying composition and sizes that may get inhaled with the air.

Atoms on the surface of a solid material miss a cohesion partner. This deficiency is aggravated in nanoparticles by the curved particle shape that produces a radial tension on surface atoms, which rises surface energy. This augments the tendency to interact with reaction partners. In addition, the total surface also increases with smaller size (Fig. 1).

When inhaled, larger dust particles tend to be held back in the upper pulmonary airways and thereafter transported by ciliated epithelium back up to the pharynx and then swallowed. Smaller particles will reach the bronchioles, and nanoparticles with a size below 100 nm tend to behave like a gas and reach the sensitive alveoli (Tsuda et al. 2013), where extremely thin alveolar cells and adjacent endothelial cells enable gas exchange. Alveolar macrophages that move between the alveolar airway surface

Parameters (relative)	Particle Size vs Number (schematic)		
Size	10 µm	2.5 µm	0.1µm
Size (visualized)			
Mass	1	1	1
Number	1	64	1 000 000
Surface Area	600	2400	60 000

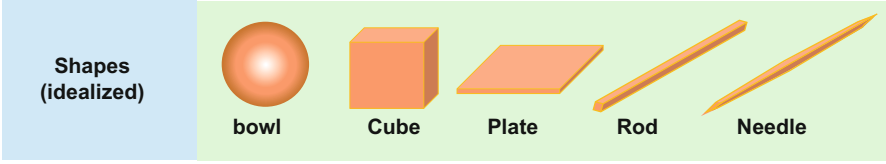


Fig. 1 Sizes and shapes of fine particles (schematic). The upper panel shows the relationship between particle size, particle number, and particle surface area. The lower panel shows some shapes of engineered nanoparticles

and the interstitial tissue phagocytize particles (Fig. 2a). The macrophages may be able to bring the particle components in solution or to store and finally deposit the particles in the lung tissue, with chronic disease risks for the lung. The overall adverse effect will depend on particle shape, number, solubility, and toxicity of its components (Wittmaack 2007; Graham et al. 2017).

Lung diseases such as metal fume-induced fever, hypersensitivity, silicosis, asbestos-related cancer, and smoking-associated pulmonary disease are well-known toxic outcomes of particle inhalation. There is also evidence for reduced life expectancy in association with high ambient particle concentration.

In order to minimize potential lung toxicity, it is important to analytically monitor particle number, size, and composition in the ambient air. Identification with particle counters, electron microscopy, and chemical analysis must be combined, in order to characterize a specific particle type and, ultimately, to avoid emissions of such particles. It is very difficult to distinguish between particles of different origin and composition in the ambient air, in water, and especially in biological fluids and tissues.

Today, engineered nanoparticles constitute a rising problem. They tend to have a well-defined composition and shape (Fig. 1, lower panel), with known scaffold and surface materials, providing technical advantages. But it remains challenging to predict the long-term toxicity of the various particle types.

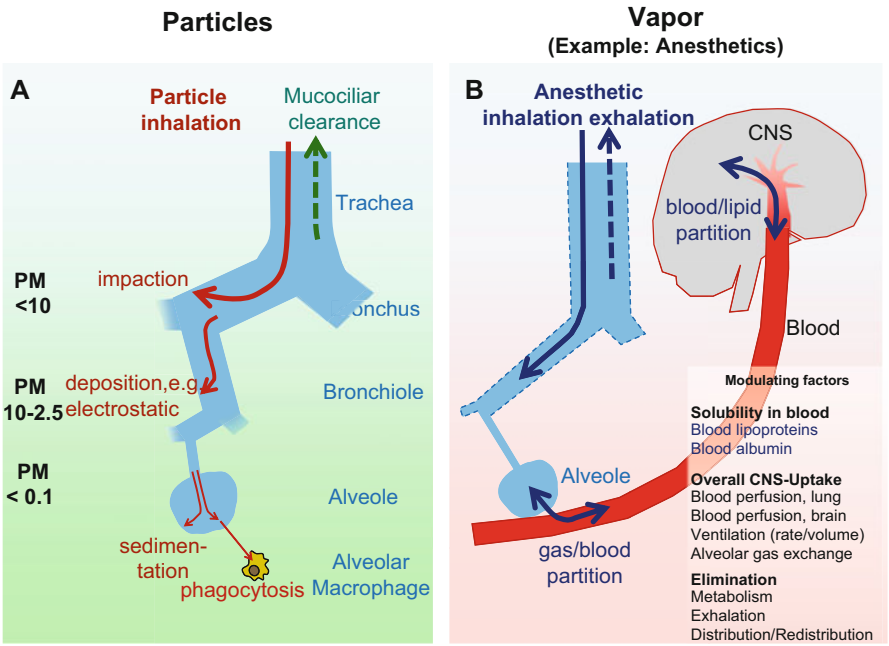


Fig. 2 Physicochemical aspects of inhalation toxicology. (**a**) Particle transport and deposition in the airways. The route of inhaled particles and their contact site with airway walls is shown in red. Their return transport by mucociliary activity in green. The sites of contact with the airway wall depend on particle size (particulate matter PM, given in micrometers). Larger particles hit the inner surface of the bronchial system by impaction and may then be removed by mucociliary clearance. Particles of intermediate size will get deeper into the airways and deposit there by hydrophilic interactions or electrostatic forces. Ultrafine particles can reach the lowest part of the airways, the alveoles. They may be phagocytized by alveolar macrophages. (**b**) An inhaled anesthetic is transported with the air stream to the alveoles. It gets absorbed into blood according to its gas/blood partition coefficient. Uptake into the brain and the narcotic effect depend on the blood/lipid partition coefficient. A variety of physiological parameters have modulating effects (see insert). Analogous partition principles apply for many volatile organic solvents

Density

The density of a material reflects the atom or molecular weight of its components.

Gas density sometimes plays a role in inhalation toxicology. For example, carbon dioxide that is formed by microbial activity in a pit may accumulate near the bottom, favored by the higher density (about 1.6-fold) of carbon dioxide compared to the mean density of air. Likewise, chlorine gas that is released from a defective tank tends to remain near the bottom of poorly ventilated rooms. When radon is emitted from building materials or from the ground, it tends to accumulate in poorly ventilated basements.

The density of fine and ultrafine particles will have an influence on the site of impaction or deposition in the airway system (upper airways, bronchi, or alveoli). The Stokes law describes sedimentation of particles in a fluid. It has been also used to describe the movement of inhaled particles in the bronchial airstream (Tsuda et al. 2013).

Solubility

Solubility is the ability of a substance (solute) to dissolve homogenously in another substance or medium (solvent). It has a key importance in toxicology. Above the critical concentration, saturation tends to occur and additional solute precipitates or results in a two-phase liquid system. The scale for solubilities ranges from very soluble to slightly soluble or insoluble.

Solubility in Water

Hydrophilic Substances

Due to its polarity and its partial ionization in hydronium plus hydroxide, water is a universal polar solvent. Chemicals that dissolve well in water are termed hydrophilic. Solid hydrophilic substances that are brought in contact with water will be subject to the process of solvation, which is the interaction of water molecules with the surfaces of the solid substance (hydration). Nonionic substances, like monosaccharide molecules, will form hydrogen bonds between the hydroxyl groups of the sugar molecule and the surrounding water molecules, thus bringing the sugar in solution. In the case of salts, water molecules induce a solvation process that leads to a dissociation of the anions from the cations, both being freely dissolved in water.

Lipophilic Substances

Organic molecules that lack hydrophilic functional groups are lipophilic (hydrophobic) substances. They are, at best, very poorly soluble in water. Intermolecular chemical forces between the polar water molecules are much stronger than those between water molecules and very lipophilic organic molecules, and thus water will "exclude" the organic molecules. Therefore, solid lipophilic substances will not dissolve in an aqueous phase, and liquid lipophilic chemicals will form a water/lipid biphasic liquid system. Depending on composition, the latter can be a system with two completely separated phases or an emulsion (like milk).

Solubility in Lipid Environment

Ethanol belongs to those substances that are miscible with water as well as with many organic solvents. Organic molecules that lack hydrophilic groups will be soluble in organic solvents but poorly soluble in water.

n-Octanol/Water Partition

To characterize a test substance with regard to hydrophilic/lipophilic properties, the substance is introduced into a two-phase system consisting of water and an organic solvent that do not mix. The n-octanol/water biphasic system (Kramer et al. 2020) is simple and very common in pharmacology and toxicology. Low amounts of the test substance are added and its distribution between the two phases analyzed and expressed as partition coefficient (Kow). Kow is a unitless number reflecting the ratio of the chemical concentration in the lipophilic phase compared to the aqueous phase. It provides no information about the maximum solubility of the agent in either phase.

Amphiphilic Substances

Amphiphilic substances have a hydrophilic and a lipophilic part. In a two-phase system, they tend to form a thin interphase. Beyond a critical concentration, some amphiphilic substances (e.g., phospholipids) may form monolayered micelles or bilayered liposomes in aqueous solution; many pharmaceutical drugs belong to this group. The various members of the membrane transporter family (SLC22a) in intestine, liver, kidney, and other organs support cellular uptake and/or cellular release of amphiphilic substances and thus have a role in their absorption, elimination, and overall kinetics. Products of phase II biotransformation, like glucuronide- and sulfate conjugates, are amphiphilic and are readily eliminated via the canalicular membrane into bile. In blood, amphiphilic substances are bound to a considerable extent to serum albumin.

Virtually Insoluble Substances

There exist substances that are very insoluble in aqueous as well as lipid environment. When ingested, such substances may pass through the length of the gastrointestinal tract unabsorbed and may be excreted unchanged. Barium sulfate is an example. Despite the toxicity of soluble barium compounds, barium sulfate can be used in gastrointestinal X-ray diagnostics because it is not absorbed.

Nevertheless, virtually insoluble substances are not generally harmless, e.g., when they are inhaled as fine particles (e.g., asbestos fibers) or when they accumulate in the environment (e.g., plastic particles or toxic metals).

Surface Adsorption

Surface adsorption is the feature of a substance in a liquid or gaseous phase to reversibly bind to the surface of solid material. It has a great role in environmental toxicology of soils, because any substance that is applied to agricultural land or spilled on the ground may leak into the ground. Depending on its volatility and solubility, it will gradually be carried with rain water and by capillary forces into

deeper layers. It will adsorb via weak chemical forces on the surfaces of soil particles, usually in a reversible manner. The extent of adsorption depends on soil composition and affects the lag time until the substance is detectable in the ground water. The Langmuir adsorption model describes the physicochemical features of adsorption (van Elteren and Budic 2005).

Atmospheric particles tend to adsorb volatile chemicals, which changes particle features and may be of relevance for the toxicity of inhaled particles.

Osmotic Activity

The organism tightly regulates the osmolarity of body fluids. The sodium-potassium pump in the cell membrane generates, within the cell, an isosmolarity of about 300 mosmolar. Erythrocytes will swell and burst due to osmotic shock when placed in water. But the human skin and mucosal surfaces are quite resistant to varying osmolarities of aqueous liquids. The luminal side of gastrointestinal epithelial cells is protected by an "unstirred layer," mucous layer, and an acid microclimate. This, together with the ion pumps on the brush border membranes, enables the cells to maintain their osmotic environment even when the osmotic activity in the intestinal lumen changes after food intake.

Osmotically active agents such as albumin and sometimes dextrose are used in intensive care to expand a shrunken blood volume and for keeping the osmotic pressure in a physiological range, thus compensating for losses of the osmotically important blood proteins.

And intravenous mannose reduces cerebral edema due to its osmotic activity. Mannose also serves as osmotic diuretic.

Volatility, Vapor Pressure, and Vapor Solubility

Volatile liquids and volatile solid substances release at their surface molecules that have the kinetic energy to evaporate into the gas phase. The tendency to evaporate at a given temperature and pressure is characterized by the "vapor pressure" of the substance, which is also relevant for the reverse reaction, the tendency of the vaporized substance to condense. Volatility and vapor concentrations are of paramount importance for exposure assessment in occupational situations, indoor air pollutions, and industrial accidents.

The equilibrium partition coefficient of a volatile substance describes its distribution between air and water according to Henry's law, which is of relevance for the pulmonary absorption and organ distribution of volatile organic chemicals. Inhalation anesthetics belong to the best studied substance groups concerning the effects of vapor pressure and vapor solubility on one side and the pharmacokinetics on the other. The well-understood principles governing the behavior of inhalation anesthetics are generally applicable for many lipophilic volatile substances of concern in toxicology.

Example Anesthetics

Most inhalation anesthetics are liquids at room temperature. They are added in a vaporizer device to the oxygen-gas stream at a certain concentration. The mixture is inhaled by the patient, and the anesthetic will be directed with the gas flow to the absorptive areas of the lung, the alveoli. There, the anesthetic substance has to travel across a space of about 0.8 micrometers consisting of the surfactant lipid layer, the alveolar cell layer, and the neighboring endothelial cell layer before entering the pulmonary capillary blood.

The blood/gas partition coefficient describes the relationship between the concentration of a gaseous substance in the lung alveoli and its concentration in blood under equilibrium conditions. It resembles the water/gas solubility coefficient (Henry's law constant) but deviates from that, due to binding of substances to blood components, notably albumin, lipoproteins, and cells. Very high solubility and strong binding of an anesthetic in blood may have the disadvantage that much of the anesthetic substance will not contribute to achieving pharmacologically effective levels. Once in the blood, the anesthetic will be carried with the circulation to peripheral organs, notably to the well-perfused brain (Fig. 2b).

The second requirement for an inhalation anesthetic is a high blood/oil partition coefficient, which enables it to cross the blood-brain barrier in sufficient amounts to induce a narcotic effect in the central nervous system. A high lipid (oil) solubility of an anesthetic agent is correlated to the potency to induce anesthesia (Pavel et al. 2020).

In conclusion, low solubility in blood is associated with rapid onset of anesthesia.

High solubility in oil is associated with a high "potency," though this type of potency seems to result from a combination of the described kinetic features and molecular mechanisms of action. Other volatile agents, like many organic solvents, follow similar kinetic rules, although their anesthetic efficiency is much lower or missing.

Example Mercury

The volatility of mercury provides a problem for human health. Beauty ointments that are traditionally used in some countries in Southern Europe to bleach the skin are composed of metallic mercury dissolved in fat. Mercury intoxications have occurred due to the application of these creams onto the skin. They can be diagnosed by mercury-related symptoms and by elevated mercury levels in human blood. It is assumed that absorption of mercury does not occur primarily via the skin route but rather after evaporation of mercury from the cream into the air and subsequent inhalation of the volatile metal by consumers and others in shared living rooms.

When mercury-amalgam fillings of teeth get old and start corroding, tiny amalgam particles will detach and be swallowed unnoticed. Because of the low solubility of mercury amalgams, gastrointestinal mercury absorption from swallowed particles will be quite low. Nevertheless, persons with several corroded amalgam fillings tend

to have increased mercury levels in their blood, compared to control groups. There is some evidence that this is caused by mercury vapor that is emitted from amalgam fillings and absorbed via the inhalation pathway.

Environmental Relevance

When metallic mercury is spilled indoors and remains undetected, it will gradually evaporate and cause mercury contamination of indoor air. Volatility is also the precondition that allows for the known environmental transport of mercury over long distances. When it settles as sediment, it may get converted by marine and soil microorganisms to methylmercury, which is lipophilic and biomagnifies as toxic substance in the food chain (Fig. 3a). Volatility also plays a role for the risk of polychlorinated biphenyls in sealing materials. Even when applied to sealing joints on the exterior of a building, PCBs that tend to evaporate when the sealing material is exposed to direct sunshine, may enter the indoor air, sediment on indoor surfaces, and thus cause a continuing PCB exposure of persons in those buildings. Moreover, long-distance atmospheric transport causes a global spreading of PCB congeners (Fig. 3b).

Viscosity

Viscosity of a toxic substance may provide serious problems. For example, sulfur mustard, a vesicant that is prohibited by the Chemical Weapons Convention, is a viscous, colorless liquid that, when a person comes into contact, sticks to skin and clothing, is difficult to remove or decontaminate, and will rapidly cause chemical burns.

Viscosity of atmospheric fine particles tends to have an influence on their agglomeration and on the adsorption of volatile substances. Inhaled low-viscosity particles enter deeper into the airways and lungs but get transported back by ciliary action more efficiently compared to particles with higher viscosity. The viscosity of air itself, which, contrary to liquids, increases with increasing temperature, also has an influence on the diffusion of fine particles.

Blood has a higher viscosity, compared to water, due to the blood cells, plasma proteins, and lipoproteins. Increased blood viscosity is a risk for circulatory incidences. Hemodilution can be used therapeutically to correct its abnormal viscosity.

Stability

Any marketed chemical is expected to remain stable during the shelf-life of the product, and higher stability is generally considered a sign of higher quality. Many materials, ranging from plastic foils to food ingredients, get stabilized by suitable additives. Pharmaceutical drugs must undergo long-term measurement of stability at

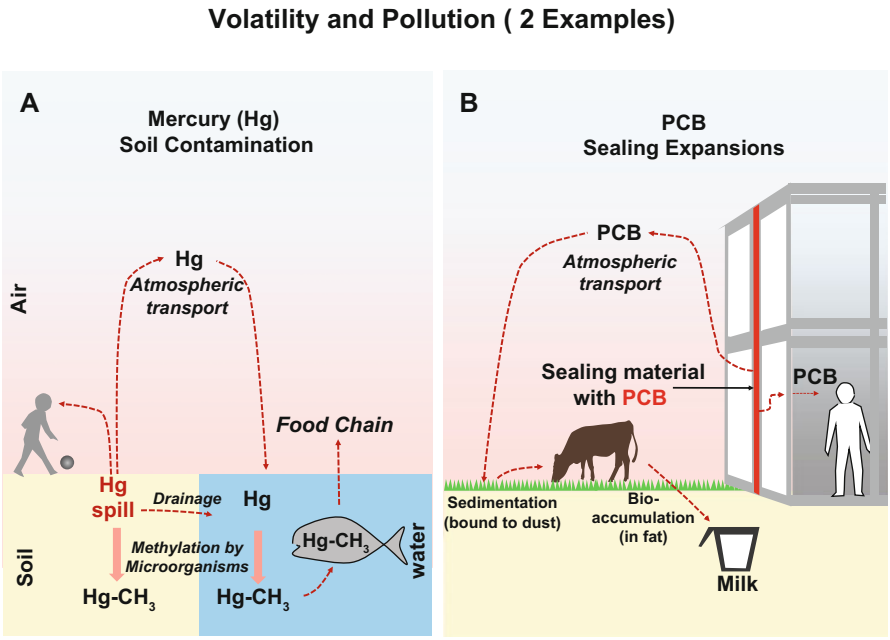


Fig. 3 Volatility of persistent substances (examples). (**a**) Metallic mercury (Hg), contaminating the soil, will evaporate with time and cause increased Hg levels in the surrounding air, a possible danger for children. Mercury vapor may also undergo longer-range atmospheric transport and sediment on water. Mercury in the soil and mercury in aquatic systems gets metabolized by microorganisms to lipophilic, toxic methylmercury that tends to get enriched in the lipid fractions of the food chain. (**b**) Polychlorinated biphenyls (PCBs) in sealing expansions of exterior building walls tend to evaporate over months and years, notably under warmer temperature conditions. PCB vapor tends to enter the building and cause irreversible indoor contamination. Volatile PCBs may also undergo atmospheric transport, and, after sedimenting on the ground, they may be taken up with the feed by cattle and appear in milk

different temperature and humidity conditions to ensure that there will be no loss of activity and no formation of toxic derivatives during storage.

Metals in the environment are physically stable. Chemically, they may undergo oxidation/reduction reactions or get chelated and thus become solubilized; some are methylated by microorganisms (e.g., mercury). Asbestos in building material is quite stable but will corrode with time and release asbestos fibers into the air.

There exist also comparatively stable organic chemicals that decompose in the soil at only a very slow rate. Examples are benzo[a]pyrene and a range of chlorinated aromatic substances including the insecticide DDT, the PCBs, or PCDDs. This high stability in the environment is the cause of ecotoxicologic problems inherent to these persistent chemicals. The so-called throwaway societies will be confronted with a world full of chemical substances, trash, and plastic that do not readily decompose in the environment.

Heat Sensitivity

Cooking, baking, and frying of food is associated with chemical changes that affect taste and appearance (e.g., Maillard reaction) but may be accompanied by formation of toxicologically relevant side products: Acrylamide occurs as a toxic side product of the reaction of amino acids with carbohydrates. When barbequing meat, mutagenic and carcinogenic substances may be formed, including benzo[a]pyrene, carcinogenic nitrosamines, and Trp-P-1/ Trp P-2, the pyrolysis products of tryptophan. Eye-irritating acrolein forms when fat (triglyceride) is heated to high temperature; first, glycerol is released by hydrolysis and then dehydrated to acrolein.

Mechanical Abrasion

The scraping off of material from a solid surface has a number of toxicological aspects, for example, in connection with release of materials from artificial joints and tooth fillings, the abrasion of allergenic nickel from accessories, or abrasion of soot from surfaces during skin contact. Standardized tests for studying abrasion resistance of materials are available.

Photochemical Instability/Reactivity

Ecotoxicology: Sunlight-induced reactivity has several toxicologically relevant aspects:

Photosmog is the result of photochemical reactions among atmospheric components, with involvement of ozone, nitrogen oxides, and aldehydes, catalyzed partly on the surface of fine particles and resulting in formation of peroxyacetyl nitrate (PAN) as one of the irritants and lacrimation-causing agents (Fig. 4a). On the other hand, photochemical destruction plays a positive role in the inactivation of polluting organic chemicals on the surface of soil.

Photosensitivity

Phototoxicity is associated with rapidly appearing skin sensation and blisters that occur, for example, after skin contact with the plant Giant Hogweed and its furanocoumarins, which penetrate the upper layer of the skin and form reactive products under the influence of light, damaging cellular molecules, including DNA, and causing photodermatitis. Sunlight-induced photosensitization and skin rashes are sometimes seen in patients taking certain drugs, such as chloroquine.

Isotopic Instability

Radioactive isotopes behave chemically (in most cases) like the nonradioactive isotopes of the elements. Potassium in the earth crust and in living cells exists mainly

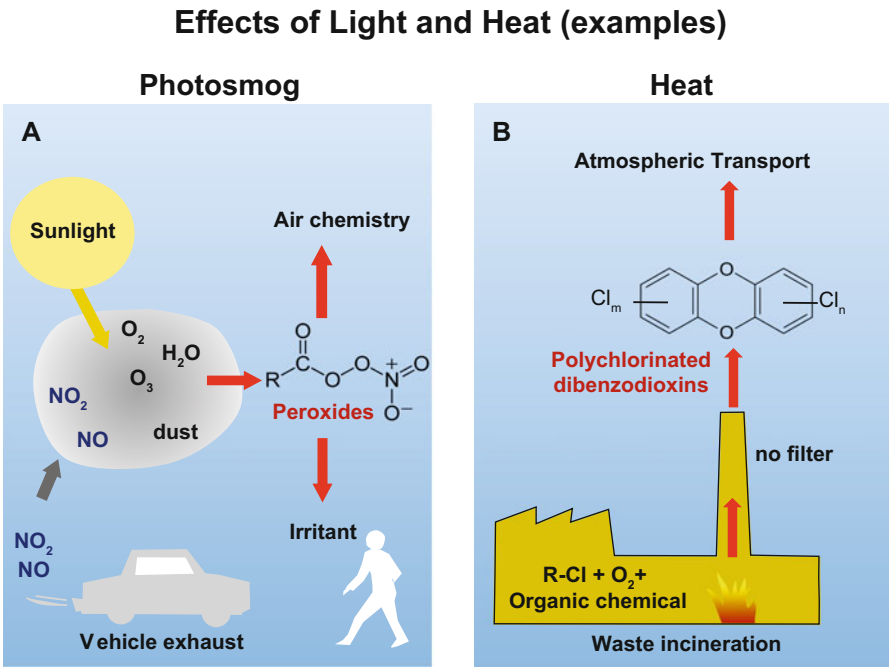


Fig. 4 Effects of light and heat (examples). (**a**) Schematic drawing showing generation of photosmog by interaction of ambient oxygen with anthropogenic nitrogen oxides (e.g., from vehicle exhausts) and other volatile air pollutants under the influence of sunlight. Peroxyacyl nitrate and related products with airway- and eye-irritating effects are formed. (**b**) Waste incineration in the presence of chlorinated chemicals such as polyvinylchloride leads to the formation of polychlorinated dibenzodioxins (PCDDs) which get emitted into the outdoor air in cases of insufficient filtration. PCDDs are persistent organic pollutants. They may undergo long-range atmospheric transport, before sedimenting

as stable isotopes (K-39 and K-41). But a very minor fraction (0.01%) is radioactive K-40, which causes some unavoidable internal background radiation.

Medicine uses radioactive elements in diagnostics and in radiotherapy. Moreover, radiolabels (C-14, H-3, and many others) introduced at specific positions of organic molecules are used in pharmacology and toxicology in order to investigate toxicokinetics and biotransformation of the agents.

Flammability

Flammability is a safety problem in itself. Some substances are subject to autoignition; others start burning at the flash point and continue burning at a temperature beyond the fire point. Apart from the direct threat for property and life, a fire has serious toxicological aspects: As soon as a substance burns, gases will develop. For the most part, the oxidation products CO_2 and CO are formed, together with smoke

of varying composition. Both gases are a danger to life, CO_2 causing dizziness and narcosis, and CO displacing oxygen from hemoglobin and producing anoxia.

Flame retardants are added to flammable materials to suppress ignition or slow down fire spreading; some of them act as radical scavengers, inactivating the highly reactive radicals formed in combustion, and thus suppress the chain reaction of heat-induced radical formation and heat production.

Plastic material undergoes thermal degradation under heat, either in the process of pyrolysis (in the absence of oxygen) or of oxidative degradation, leading to formation of volatile and particulate emissions into the air. When plastic material burns, CO_2 and CO are common combustion products. Burning organic material also releases irritants such as aldehydes. In addition, cyanide (HCN) may be produced from burning wool and polyurethane. The gases are acute health hazards. When polyvinylchloride (PVC) is present in a fire, hydrochloric acid (HCl) as well as polychlorinated dibenzodioxins (PCDD) tend to be formed. Emitted PCDD will subsequently sediment in the environment and may constitute a chronic exposure risk (Fig. 4b).

And when a larger container with a flammable toxic agent explodes in an incident, released gases and volatile substances may cause contamination and fatalities in the region, as was the case in the Bhopal accident in India, in 1984.

Acute exposure guideline values (AEGL) have been defined that are useful in such incidences to decide about adequate protection measures for the exposed population.

Kinetics and Reaction Types

Toxicokinetics

Toxicokinetics is the field that describes the time courses of appearance and disappearance of an administered substance in body fluids. The overall process involves rates and extents of absorption, distribution, metabolism, and elimination (ADME). Elimination kinetics describes the disappearance of the xenobiotic after it has reached its peak concentration in a body fluid. The decrease typically follows first-order kinetics. Biphasic and triphasic elimination curves may result from involvement of multiple metabolic pathways and elimination routes (bile, urine). As rule of thumb, one can often assume that a substance is quasi-eliminated after five half-lives (corresponding to 97% eliminated). Vice versa, when a substance is repetitively (e.g., daily) administered, 50% of maximal blood level will be reached after one half-life.

Ethanol is an exception, because its high levels in body fluids after a drink saturate the ethanol metabolizing enzymes, with the consequence that ethanol elimination is not exponential – but linear with time (zero-order elimination kinetics). ADMET (absorption, distribution, metabolism, elimination, toxicology) is a tool set, applying physiologically based toxicokinetic modeling in order to make predictions about toxicological relevance (Pozzan 2020).

Interaction Types

The physicochemical subdiscipline "reaction kinetics" deals with the time courses of chemical reactivity. The subdiscipline "thermodynamics" deals with the energy requirements and the equilibrium state of a reaction. Both are important aspects of the molecular mode of action of toxic agents.

The mechanisms of adverse chemico-biological interactions depend on the chemical substance and its biological interaction partner. Highly reactive chemicals like mustards may covalently bind to biomolecules and destroy molecules and tissues. Toxic metals tend to bind to sulfhydryl groups and inactivate proteins. Free radicals that may arise during biotransformation of some substances or in the course of oxidative stress may covalently bind to biomolecules or initiate the process of lipid peroxidation, causing defects in lipid membranes. Such interactions are usually irreversible and analytically identifiable in the form of abnormal protein adducts, breaks in macromolecules, or lipid peroxidation markers.

Non-covalent chemico-biological interactions between the toxic substance and a ligand binding site of a receptor or enzyme are usually reversible, following the principles for inhibitors in Michaelis-Menten kinetics.

Only few toxic agents are suicide substrates that bind covalently to the active center of an enzyme; organophosphates belong to this group. Ligands of biological receptors tend to have more than one interaction site with the target protein, such as a lipophilic weak (van der Waals) bonding with a hydrophobic pocket plus electrostatic interactions plus hydrogen bonding, which together enable a precise steric positioning of the ligand within the binding site of the protein. Models about toxic modes of action of a substance tend to be a matter of scientific dispute before a decision about their relevance for toxicological risk assessment is made.

Chelation is a type of reaction, in which several weak interaction sites in an organic molecule bind a metal-cation in a cage-like manner. Chelators are used to treat patients who were diagnosed with metal intoxication; they mobilize the metal and enforce its elimination via the kidneys. Chelators are also used technically, e.g., to keep calcium soluble in washing machines in order to protect the machine and the textile. When entering environmental water systems, chelators have the tendency to mobilize toxic metals, but they also decrease essential metals required by aquatic organisms. Therefore, chelators have a relevance for aquatic toxicology.

In summary, physicochemical features of a substance are of great relevance for its characterization as a unique substance, its analytical detection, and its safety profile. The physicochemical properties provide essential and unique information, required for risk assessment, notably in the areas of exposure assessment, toxicokinetics, and ecotoxicology. They are a necessary but not a sufficient component in all kinds of toxicological risk assessment.

Cross-References

- ▶ Assessment of Mixtures: Bridging Principles and Other Approaches
- ▶ Characterization of Physicochemical Parameters in Toxicology
- ▶ Intrinsic Toxicity of Substances: Aspects for Risk Assessment
- ▶ Medical Aspects of Traffic Exhaust Regulations
- ▶ Microplastics: A Novel Suite of Environmental Contaminants but Present for Decades
- ▶ Toxicodynamic Models
- ▶ Toxicokinetic Tests

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Intrinsic Toxicity of Substances: Aspects for Risk Assessment

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Michael Schwenk and Reinhold Burr

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M. Schwenk (✉)
Tübingen, Germany

Formerly: Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

R. Burr
General Practitioner Practice, Hirrlingen, Germany

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Abstract

Toxicity is an inherent feature of many different types of chemical substances, such as acids/bases, heavy metals, chlorinated molecules, organic solvents or protein-toxins. Specific chemical structures tend to be associated with characteristic modes of chemico-biological interactions and respective adverse effects in the living organism. The principles of "intrinsic toxicity" are of considerable importance for risk assessment, notably when toxicity data of known substances are missing or incomplete. In cases of intoxication with an unknown substance, the pattern of clinical symptoms may be a key, guiding to the type of substance causing such adverse effects. This contribution illustrates the concepts of intrinsic toxicity from the viewpoints of both, chemical structure and biological effects.

Keywords

Mode of action · Chemical · Cytotoxicity · Repair · Target organ · Sensitive individual

Introduction

Intrinsic toxicity reflects the ability of a substance to access biological targets (toxicokinetics) and to interact with target biomolecules and deteriorate biochemical pathways with an adverse outcome for health (toxicodynamics).

The term "intrinsic toxicity" has sometimes been used in a narrower sense to describe endogenously formed toxic products such as free hemoglobin (Simoni et al. 2009) or glucose-adducts (Szwergold 2005). Herein, the term "intrinsic toxicity" is used in a wider sense, considering structural features that are characteristically associated with a mode of adverse action, and providing examples for organ-related outcomes. It refers to the questions: What makes a substance toxic and how does an organism respond?

Intrinsic toxicity of a substance can be described in different ways: By structural features of the substance and its functional groups, by the biomolecular mode of action, and by the type of adverse effect in the living organism. Intrinsic toxicity of a substance is one cornerstone of overall toxic action, the other ones being intrinsic responsiveness of target organs, exposure, and dose (Fig. 1). Knowledge of the principles of intrinsic toxicity is essential for risk estimation and a key to predict or estimate adverse effects when no toxicity data are available. It is also the basis for the analogy principle and the read-across method in risk assessment. Nevertheless, each substance is unique, and the complete set of toxicokinetic and toxicodynamic information is needed to fully characterize its intrinsic toxicity in detail.

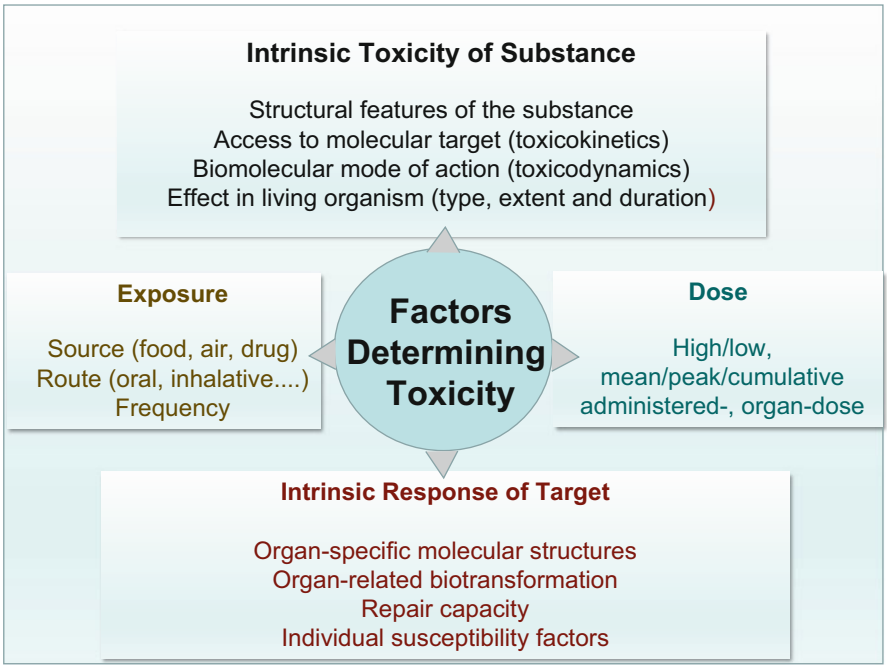

Fig. 1 Intrinsic toxicity as major factor determining toxicity. Four parameters affect the outcome of a toxic interaction: intrinsic toxicity of the chemical, type of exposure, dose, and the specific response of the target organism

Structure-Related Intrinsic Toxicity

Intrinsic toxicity features are dependent on the structural components of a compound. Conceptually, one may consider a chemical substance as consisting of a core structure (atom, molecule, or particle) of a certain size, to which functional groups and side chains are attached (Fig. 2).

Size, lipophilicity, and cationic/anionic charge largely decide about absorption, distribution, and cellular uptake. Oxidation-sensitive side groups of the substance decide about the route and velocity of phase I metabolism, including generation of reactive intermediates with cytotoxic and genotoxic potential

Toxicokinetic and Toxicodynamic Aspects

The structural composition is important for both toxicokinetics and toxicodynamics.

Toxicokinetics: In order to reach its target organ, a xenobiotic must get absorbed and carried with the circulation to its target cell. A long lipophilic side chain, introduced in a molecule, may enable it to cross organ barriers and biological

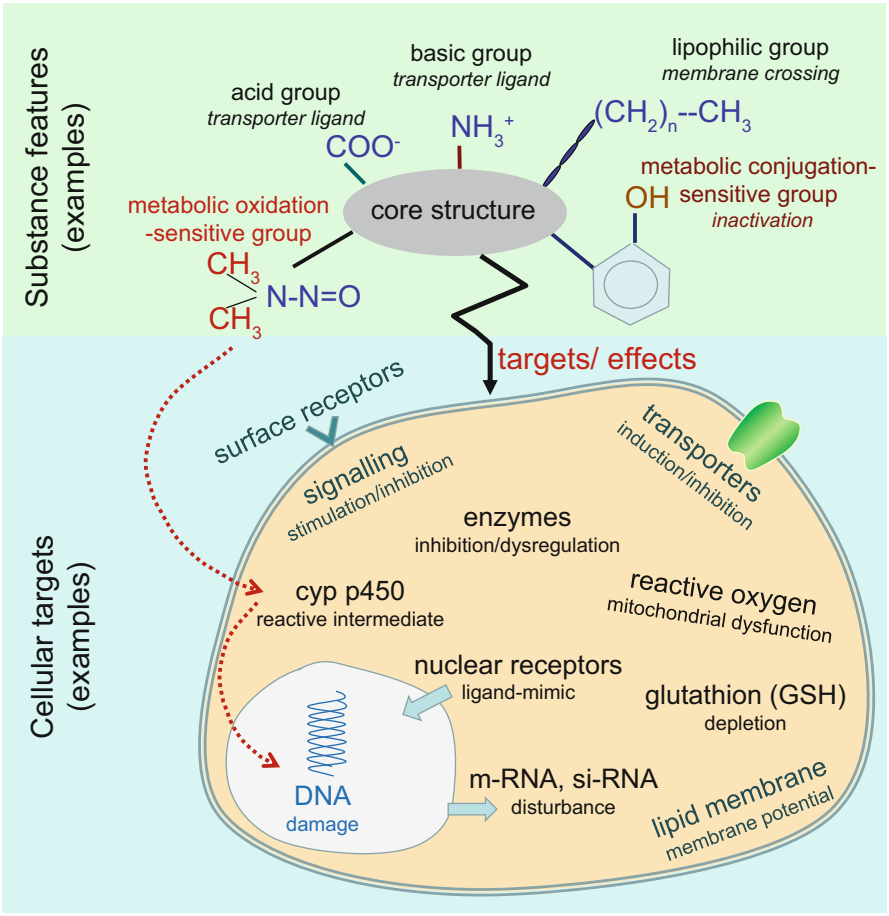

Fig. 2 Substance features and common cellular targets. Schematic drawing of the core structure and various types of functional groups of a model chemical substance (upper panel). Major cellular and biomolecular targets for toxic actions are shown in the lower panel

membranes. Carboxylic groups or amino groups may enable the substance to be transported by one of the families of membrane transporters, such as organic anion transporters (OAT), organic cation transporters (OCT), or ATP-dependent exporters (ABC-transporters). Some of the transporters support cellular uptake and others cellular elimination, and the overall process is an important determinant for the half-life of the active substance within a cell type.

Ingested dietary proteins are degraded in the gastrointestinal tract by proteases. In most cases, only short peptides and amino acids can get absorbed via the respective transporters. However, several toxin proteins have structural features that make them intrinsically resistant to such degradation and even allow them to cross the gastrointestinal barrier by endocytosis or related processes, favored when the epithelial lining is not completely tight.

Most xenobiotics undergo biotransformation mostly in the liver but also in extrahepatic drug-metabolizing organs like kidneys, gastrointestinal tract, and lung. Often hydroxyl groups are introduced by one of the cytochrome P450 isoenzymes through a mechanism whereby oxygen is transferred to the substrate in mixed-function oxidase reactions, involving NADPH as cofactor and yielding one or several different phase I biotransformation products. Phase I metabolism normally detoxicates xenobiotics; however, some xenobiotics are converted to reactive (intermediate) products.

Xenobiotics with a preformed hydroxyl group (e.g., naphthol) and hydroxylated phase I products are typically further converted in phase II biotransformation reactions (conjugation) often with glucuronic acid and sulfate. In most cases, conjugates tend to be less toxic. They exit liver cells via membrane transporters into bile or, alternatively, into blood with subsequent urinary elimination.

Toxicodynamics: The chemical reactivity in combination with the three-dimensional architecture of a substance is the basis for toxicodynamics. Figure 2 (lower panel) shows examples of molecular targets within a cell. Many toxic mechanisms are based on non-covalent interactions, often with an enzyme, a receptor, or membrane transporter. Such non-covalent interactions are usually reversible and directed quite selectively against a specific target molecule. Other toxic substances, such as corrosive agents or reactive metabolites of phase I biotransformation, tend to interact covalently and less selectively with surrounding biomolecules, producing cytotoxic damage, often including genotoxic DNA adducts and breaks, or initiate a destructive pathway like glutathione depletion, oxidative stress, or lipid peroxidation.

Though each chemical has its individual toxic footprint, there exist structural patterns that are associated with a specific toxic interaction. The following section provides some examples.

Corrosive Substances

Ingestion of corrosive substances usually leads to a medical emergency (Kalayarasan et al. 2019). Strong **acids** and **bases** have an acute globally destructive effect on living tissue. On the molecular level, acids and bases interact with the functional groups of macromolecules and destroy their repulsive surface layer and intramolecular folding, thus resulting in denaturation and agglomeration of macromolecules. This results in cell death (necrosis) and destroys the fine structure of surrounding tissue. Strong bases tend to dissolve the denatured tissue and thus produce deeper lesions (liquefactive necrosis) that will later transform into scars and irreversible damage. Acids precipitate proteins in a way that may result in a protective layer of denatured material, thus self-limiting further destruction of the tissue below (coagulative necrosis).

The stomach provides an exception. The gastric proton pumps may generate an acidity of less than pH 2 in the gastric lumen. A healthy gastric and duodenal mucosa has a surprising ability to protect itself from self-destruction by secreting neutralizing bicarbonate and mucus to the mucosal surface. When this protection fails, an

ulcer may develop, and in case of a pathologic reflux of gastric acid to the esophagus, a reflux esophagitis may result.

The eyes are predisposed to damage by acid or alkaline spills, and the oral cavity, pharynx, and esophagus are at danger, when corrosive substances are swallowed.

Volatile acids, such as formic acid or concentrated sulfuric acid, are relevant as inhalative toxicants. When inhaled, they dissolve in the aqueous phase of the airway mucosa, which they acidify, thus causing irritation and damage. Likewise, volatile alkaline substances like ammonia dissolve in the aqueous phase of the airway mucous membrane, making it more basic, and are very aggressive to the airways when inhaled.

A different mechanism of corrosivity is exhibited by sulfur **mustards** (Ghabili et al. 2011), nitrogen mustards, and a number of organo-arsenic compounds (e.g., Lewisite). These substances are chemically very reactive; bind covalently to glutathione, protein, DNA, and other types of biomolecules; and thus are universally corrosive to exposed skin, airways, and, in general, any tissue. These substances are banned internationally by the Chemical Weapons Convention.

Numerous regulatory restrictions and warnings exist for corrosive substances. International pictograms are available to label containers that hold corrosive substances (GHS 2020).

Short-Chain Aldehydes

Various short-chain aldehydes are volatile reactive chemicals with the potential to bind to biomolecules, thus irritating exposed body surfaces and airways already at low dose (LoPachin and Gavin 2014). Repeated exposure may lead to a sensitization, either via neurogenic inflammation or via allergic sensitization. An example is formaldehyde (Bernardini et al. 2020), which once constituted a widespread air problem indoors, often due to its release from urea formaldehyde foams used for insulation of houses, before stabilizers were commonly used that suppress formaldehyde release.

Short-Chain Chlorinated Solvents

Carbon tetrachloride, chloroform, methylene chloride, trichloroethylene, and related halogenated aliphatic organic substances are volatile, lipophilic chemicals. They have a long history as solvents, vinyl chloride in addition as polyvinyl chloride precursor. Due to their lipophilicity, inhalation at the workplace causes stupefying and narcotic effects on the brain and side effects on the nervous system.

Many chlorinated solvents are readily biotransformed in the liver. This is usually accompanied by abstraction of a chlorine atom or, in the case of vinyl chloride, formation of an epoxide. Biotransformation tends to be associated with formation of reactive intermediates that are cell toxic and cause acute liver cell damage. Dichloromethane is metabolized to carbon monoxide which may cause carbon

monoxide intoxication. One pathway of trichloroethylene biotransformation is the conversion to trichloroacetic acid, a strong acid.

Chronic carbon tetrachloride exposure causes liver cirrhosis and liver cancer. Chronic vinyl chloride may cause a tumor of liver vascular walls (angiosarcoma). Dichloromethane is carcinogenic to the kidney. Additional targets exist. For example, chloroethane makes the heart more sensitive to catecholamines and arrhythmias.

Volatile short-chain chlorinated solvents tend to have a long half-life in the atmosphere and to affect air chemistry negatively.

In general, the intrinsic toxicity of these substances has several roots: Their small molecular size and the lipophilicity favor inhalative absorption and uptake into the brain, causing acute and chronic neurotoxicity. The genotoxicity of reactive intermediates constitutes a cancer risk. Many regulations exist, to restrict exposures of workers and the general population and to minimize release into the environment, though the latter is a continuing matter of discussion.

Nitrosamines

Nitrosamine or *N*-nitrosamine is the name for substances with the general formula R1R2(*N*-*N*=O) where R1 and R2 represent alkyl or aryl residues which are attached to a *N*-nitroso group. When taken up in the body, most nitrosamines are metabolized by drug-metabolizing phase I enzymes. This generates instable intermediates, notably methyl cations (Fig. 3).

The lower panel shows the reaction sequence for dimethylnitrosamine. After hydroxylation by CYP2E1, the product decomposes spontaneously to finally form formaldehyde and a methyl cation, which is highly reactive, causing cell damage and genotoxicity

The reactive intermediates bind covalently to neighboring biomolecules, thus inactivating them. High acute doses of dimethylnitrosamine may cause fatal liver cell necrosis. Medium acute doses do not cause widespread necrosis but are associated with genotoxicity and may cause, after a latency period, appearance of preneoplastic islands in the liver and finally cancer of the liver and other organs (Robles 2014).

In addition, when secondary amines are ingested and get in contact with nitrite in the acidic environment of the stomach, there is the probability of endogenous nonenzymatic formation of nitrosamines (Fig. 3, upper panel). Pharmaceutical drugs that carry a secondary amine group need to undergo tests that make sure that they are no precursors of nitrosamine formation. Nitrosamine impurities in pharmaceutical drugs have occurred, e.g., in sartan-, metformin-, and ranitidine-containing products (WHO 2019). This is a danger signal for regulatory agencies that has led to warnings and recalls. According to the described synthesis principle, nitrosamines may also be formed as unwanted side products during some types of food processing. Threshold limit values for nitrosamines have been defined for various foods and beverages, e.g., certain types of dark beer often according to the ALARA principle (as low as reasonably achievable), or “below detection limit.”

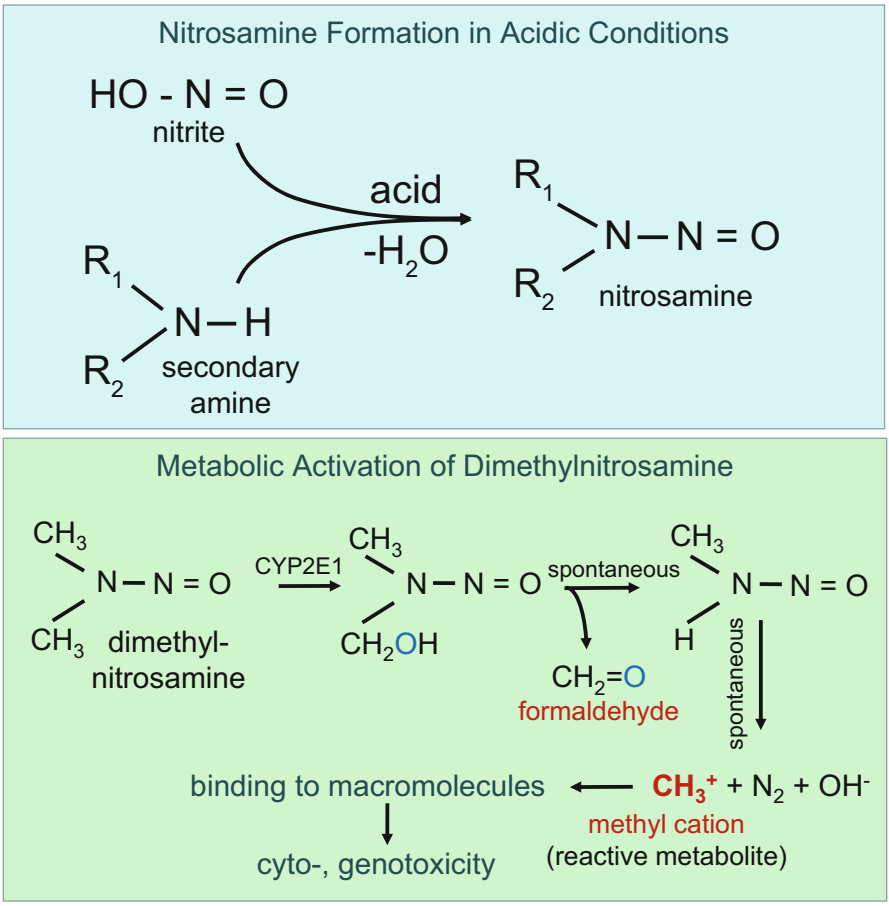

Fig. 3 Nitrosamine: formation and metabolic activation (schematic drawing). The upper panel shows the reaction in acidic conditions of nitrite with a secondary amine (characterized by two organic residues) to form a nitrosamine. Nitrosamine formation may occur in the acidic environment of the stomach, when secondary amines (components of some nutrients, some drugs) react with nitrite

N′-Nitrosonornicotine is a major tobacco-specific nitrosamine. It is generated by nitrosation of nicotine during tobacco curing, processing, and smoking.

Most nitrosamines are mutagenic and carcinogenic. The intrinsic toxicity of nitrosamines is due to the formation of reactive chemical species.

Organophosphates

Acetylcholine is a major neurotransmitter substance in the brain, peripheral nervous system, and neuromuscular junctions. To terminate each pulse of acetylcholine-

mediated neurotransmission, acetylcholine released into the synaptic space gets inactivated rapidly by the enzyme acetylcholine esterase (AChE), which cleaves the ester bond of the molecule. In this catalytic process, the choline moiety binds to the "anionic site" in the catalytic center of the enzyme and the acetyl part of the molecule to the "esteric site." Subsequently, acetylcholine gets cleaved. Choline is released and the acetate-enzyme bond gets hydrolyzed.

Organophosphate compounds bind with their phosphorus to a serine residue of the esteric site of the enzyme, resulting in release of a side group from the organophosphate. The remaining enzyme adduct does not dissociate readily. The resulting blockade of the enzyme is initially reversible, but later it may become irreversible during an "aging process." Variants of the organophosphate side chains have been developed to enhance target specificity of biocidal organophosphates and to optimize their decomposition rate in the environment. Some (e.g., parathion) are used as insecticides. Molecular modifications led to highly efficient irreversible inhibitors of the enzyme that are potential warfare agents (e.g., sarin), prohibited by international convention.

Malathion lotion is approved in various countries for application on human skin to fight head lice. On the other hand, many fatalities worldwide are due to wrong usage or suicidal intake of organophosphate insecticides (Robb and Baker 2020). Warning pictograms and labels are of little help in the case of suicidal intention but very crucial in other cases and, together with recommendations for careful and correct handling, help to avoid intoxications.

Phenylethylamines

Phenylethylamines are a class of psychoactive substances that share a common chemical core structure. They occur as endogenous neurotransmitters such as noradrenalin and dopamine or as plant-derived active substances such as ephedrine. Phenylethylamine analogues have been developed as therapeutic drugs (e.g., amphetamines) but also include illegal psychoactive drugs of addiction. The "cathinones," characterized by a keto-group, represent a large and increasing group of novel psychoactive substances found on black markets, responsible for intoxications of drug users in many countries.

Any substance with phenylethylamine structure, which is seized by the police on the black market, is considered as a signal for clandestine use and potential intrinsic toxicity (depending on dose). Forensic experts tend to apply the analogy principle in situations, when the chemical identity of the substance has been determined, but no pharmacological and toxicological data are available.

Aromatic Chlorinated Substances

Aromatic chlorinated substances became known as persistent organic pollutants (POPs) of high environmental concern. This group of substances includes insecticides like

dichlorodiphenyl trichloroethane (DDT) and technical oils containing mixtures of polychlorinated biphenyl congeners (PCBs), as well as a large group of congeners of polychlorinated dibenzofurans (PCDF) and polychlorinated dibenzodioxin (PCDD). The latter are not produced for technical applications but may occur as side products during synthesis of the wood preservative pentachlorophenol and during incineration processes when chlorine is present, notably waste incineration. Many of the congeners of PCBs and PCDDs were found to be very resistant to metabolism, thus cumulating in human fat tissues over many years. They are also quite resistant to environmental breakdown and thus bioaccumulate in the food chain, notably in animal fat, milk, egg yolk, and the final consumer, the humans, notably in mother's milk. The congeners have on the whole the same toxic endpoints but different strengths of adverse effects (see Sorg 2014). Toxicity equivalency factors (TEFs) are assigned to each congener, thus allowing to calculate the total risk, on the basis of the measured exposure dose of each congener. The use of DDT as insecticide was stopped almost worldwide not least because it cumulates in the environment and is suspected of tumor-promoting activity.

The intrinsic toxicity of many chlorinated aromatic POPs is due to their accumulation in the food chain, very long half-life of many congeners, and a combination of various serious adverse effects. Regulation of POPs for the protection of human health is covered by the international Stockholm convention.

Many polycyclic organic substances are inducers of a number of drug-metabolizing enzymes, among them cytochromes P450 and glucuronosyltransferase. Aromatic hydrocarbons like benzo[a]pyrene and methylcholanthrene and chlorinated heterocyclic organic compounds, like PCDDs and DDT, are ligands of the arylhydrocarbon receptor (AhR). They bind to the receptor in the cytoplasm, which then associates with the protein ARNT (aryl hydrocarbon receptor nuclear translocator). The complex translocates to the cell nucleus and activates transcription of specific genes (Nebert, 2017).

One of the best known effects of AhR-receptor binding is the induction of cytochrome P450 in the liver. Animal treatment with such inducers causes an up to about eightfold increase of cytochrome P450 isoforms.

Various pharmaceutical drugs (classically phenobarbital) or ethanol may induce cytochrome P450 as well, however with different isoenzyme expressions compared to chlorinated POPs.

Induction of xenobiotic metabolizing enzymes has two types of consequences: First, it increases the rate of biotransformation of xenobiotics and therefore reduces circulating levels of xenobiotics, therapeutic drugs, and some hormones. Second, it accelerates formation of reactive intermediates of procarcinogens and may increase the risk of genotoxic damage. The "human cytochrome P450 (CYP) n-fold induction in vitro test method" (OECD 2014) provides further information.

Lectins

Lectins are proteins that have a high affinity to glycoproteins on the outer surface of cell membranes. Lectins occur as natural ingredients in many plants. When lectin-rich beans are ingested without preceding heat denaturation, the lectins bind to

intestinal epithelial cells and cause gastrointestinal distress. One of the most toxic lectins is ricin, found in castor beans. When ingested, it gets in contact with mucosal surfaces and disturbs the exposed cells. Ricin is at the same time a nonbacterial AB toxin that can be taken up by endocytosis into cells, where it inhibits protein biosynthesis. Ricin is in the list of potential warfare agents.

AB Toxins

AB toxins are bacterial toxins containing two protein subunits (Odumosu et al. 2010; Kumar et al. 2019). The B subunit (binding) binds to cell surface glycoproteins and enables the toxin to enter a cell via receptor-mediated endocytosis. In the target cell, the A subunit (active) exerts its adverse effect by inhibiting or activating a vital cellular pathway often due to its intrinsic enzymatic activity, thus causing cellular dysfunction and organ failure. Though AB toxins have this general mechanism in common, the various toxins differ with regard to their routes to their target cell types, molecular endpoints, and symptoms of intoxication. Here are some examples:

Cholera toxin is released during cholera infection in the intestinal lumen and taken up by intestinal epithelial cells, where it switches on the production of cyclic-AMP (c-AMP). This activates outward directed ion pumps, followed by secretion of large amounts of water into the intestinal lumen, thus causing diarrhea and possibly death due to extreme water- and electrolyte loss.

Quite differently, the AB toxin of botulinum known as botulinum neurotoxin (BoNT) specifically acts in neurons (Pirazzini et al. 2017). When BoTN A is inadvertently ingested with toxin-contaminated food, it is largely resistant to digestion by proteolytic enzymes. It is taken up via endocytosis in the intestine, and once in the blood and lymphatic system, it gradually enriches on high-affinity binding sites (BoNT-receptor) on presynaptic membranes of cholinergic neurons, mainly on the neuromuscular junctions, to which the B subunit (heavy chain) binds firmly. The two subunits of BoNT are then internalized into vesicle cavities, which recycle to the presynaptic space. On their way, the vesicles get acidified by a proton pump activity, the bisulfide bridge between the two BoNT subunits dissociates, and the A subunit (light chain) finds access to the cytosol. There it exhibits a metalloprotease enzymatic activity, thus disabling proteins (SNARE proteins such as synaptobrevin) required to fuse the synaptic vesicle with the plasma membrane and to release acetylcholine. Thus, BoNT arrests further release of acetylcholine and therefore interrupts this neurotransmission (Fig. 4).

Botulinum toxin is considered to be the most toxic substance known and listed in international agreements of prohibited chemical weapons. Nonetheless, it can be used therapeutically under strictly controlled conditions to relax spasms and tensions of locally treated small muscles.

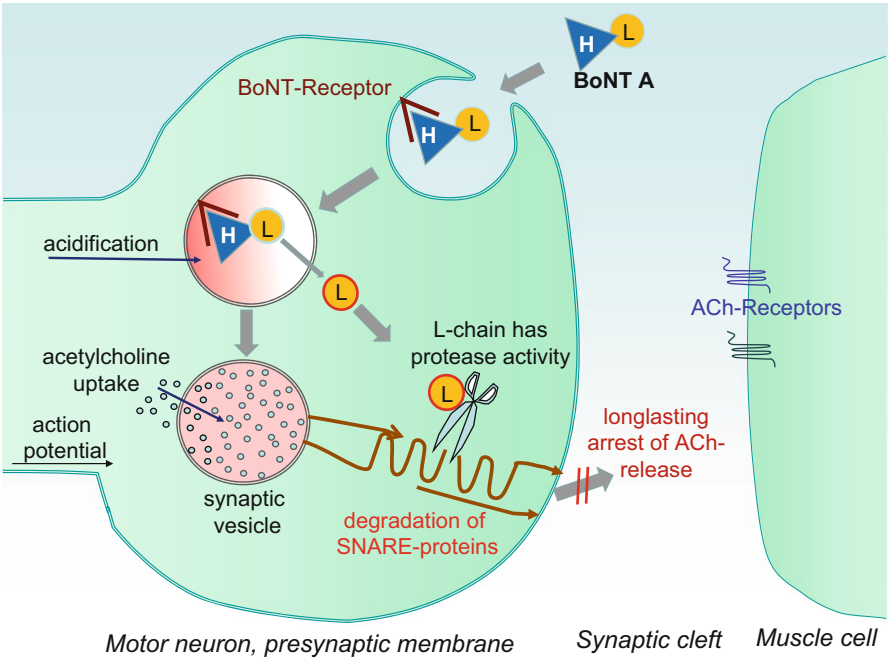

Fig. 4 Simplified schematic drawing of a cholinergic motor neuron synapse and the effect of botulinum neurotoxin A (BoNT A). Botulinum neurotoxin (BoNT A) is a bichain protein, consisting of a heavy chain (H) and a light chain (L). The H-chain shows high binding selectivity for receptors on presynaptic membranes of motor neurons. The BoNT-receptor complex is endocytosed. The vesicle gets acidified by an ATPase, which induces release of the L-chain into the cytosol. The acidified vesicle takes up acetylcholine (ACh). The cytosolic L-chain has protease activity; it cleaves and inactivates SNARE-proteins that are required to fuse the synaptic vesicles with the presynaptic cell membrane. This way, BoNT A causes a long-lasting arrest of ACh release and inhibition of muscle activities

Tetanus toxin exhibits a similar molecular mechanism of action on neurons but is transported in a different way by retrograde axonal transport to the spinal cord, where it inactivates inhibitory interneurons, resulting in spastic paralysis.

The A-unit of diphtheria toxin has ADP-ribosylation enzyme activity that is directed toward the ribosomal elongation factor EF-2. Diphtheria toxin thus inhibits protein synthesis, causing potentially life-threatening acute disease.

Anthrax toxin consists of three subunits. The B subunit is responsible for cellular uptake of the protein complex. One of the two A subunits (lethal factor) has peptidase activity that inactivates MAPKK enzyme, a key regulatory kinase involved in stress-mediated cell survival. The other A subunit possesses adenylate-cyclase activity and disturbs cellular signaling. Working together, they cause cell death (apoptosis).

AB toxins share a number of intrinsic toxic features, which makes them very different from other substances. They are well suited as model to demonstrate that

intrinsic toxicity comprise both kinetic and dynamic aspects: (1) escaping complete intestinal digestion, (2) targeting specific cell types with high affinity, (3) resistance to cellular proteolysis, and (4) exhibiting highly selective destructive enzyme activity.

The field of AB toxin-producing organisms is within the responsibilities of microbiology. It includes prevention of growth of the respective bacteria (e.g., cholera, botulinum) in the human environment, active vaccination to prevent infections (e.g., diphtheria), and antitoxin treatment of intoxicated individuals.

AB toxins can be expected in the future to serve as an archetype for the development of novel therapeutics that target specific cell types and molecular functions.

Dust, Fine, and Ultrafine Particles

Airborne dust consists of particles of varying sources, diameters, and chemical composition. Larger dust particles tend to sediment according to their mass and size. Ultrafine particles and nanoparticles tend to behave like gas. House dust is rich in tiny textile fibers, dander, and hair. It also contains various amounts of allergens of house mites and mold. Street dust contains abrasions of tires, brakes and road materials, vehicle exhausts, and more.

When airborne dust is inhaled, penetration depth is inversely correlated to particle size. Larger dust particles sediment on the walls of the nose, pharynx, and upper section of the bronchial tree and will usually be cleared by the ciliated epithelium. Inhaled fine particles move deeper, ultrafine particles, and nanoparticles can reach the deepest, oxygen-absorbing tissue of the lungs, the alveoli. In the lung alveoli, ultrafine particles may initially get phagocytized by alveolar macrophages. Poorly soluble particles that are not cleared from the lung tend to cause chronic adverse lung effects, sometimes pulmonary cancer.

There is good evidence that the number of inhaled particles is more relevant for pulmonary toxicity than particle mass (Kwon et al. 2020). However, many additional parameters will co-determine toxicity such as particle shape, surface area, surface charge, solubility, degradability, and presence of soluble toxic components (see Oberdörster and Kuhlbusch 2018). There seems to be an association between airborne particles and cardiovascular disease.

Risk assessment with regard to chronic toxicity is difficult even for single particle types, such as crystalline silica. It is even more difficult with regard to multicomponent particles formed in combustion processes or urban dust that is composed of different particle types from many different sources. Due to their surface activity, these tend to agglomerate and/or undergo photochemical reactions with oxygen, nitrogen oxides, and volatile chemicals in the air. A detailed characterization of urban airborne particles will have to include a battery of methods, such as cascade impactor, particle counters, electron microscopy, and analytical chemistry.

The common intrinsic toxic characteristics of ultrafine particles are (1) airways and lungs as primary targets, (2) inflammation in the airways, and (3) pulmonary accumulation of particles.

Metals and Metal Compounds

Metal toxicology is an extraordinary complex field (see also: Nordberg et al. 2014; Kozlowski et al. 2014) for several reasons. First, a number of metals are essential components of the human organism and can hardly get classified as inherently toxic agents, although elevated levels of essential macrominerals and trace minerals may cause disease under certain pathologic conditions. Second, metals of higher order in the periodic system exist as inorganic and organic compounds, in different oxidation states, each having specific toxicokinetic and toxicodynamic features. Third, it is sometimes not clear if metalloids, like arsenic, should be considered as metals or nonmetals. Fourth, the route of uptake (oral, inhalative, dermal) affects the severity and type of toxicity. Fifth, various metals are stored in the organism over a longer period and thus may accumulate, often unnoticed.

Essential Metals

Within the organisms, levels of the macrominerals sodium, potassium, calcium, and magnesium are well kept within a physiological range. Nevertheless, due to increased intake or biological dysregulation, body overloads may occur causing disorders or disease, e.g., sodium (edema and hypertension), potassium (cardiac arrhythmias), calcium (decreased neuronal activity), or magnesium (confusion and others).

Likewise, the levels of trace minerals (e.g., iron, manganese, copper, and zinc) are tightly controlled in the organism. This is extremely important, as seen in the case of iron.

Free iron is toxic to cells under oxidative stress, because $iron^{2+}$/$iron^{3+}$ cycling is associated with the formation of aggressive hydroxyl radicals according to the Haber-Weiss reaction. The organism has a number of mechanisms to keep levels of free iron in a narrow range: Intestinal iron absorption is downregulated when sufficient iron is present. Nonfunctional iron in blood plasma is tightly bound to transferrin. Iron uptake into cells occurs via iron-specific membrane transporters in a hepcidin-regulated manner. Excess iron is stored in the core of iron-rich ferritin particles, surrounded by a protein shell. Functional iron in the heme group of hemoglobin and cytochromes is tightly bound as central atom in the porphyrin complex.

Presently, it is undecided whether chromium is an essential mineral. Here we focus on a different aspect, namely, the effect of the oxidation state of chromium on toxicity. Chromium (VI) compounds are carcinogenic (Wang et al. 2017). They may enter cells via the sulfate-anion membrane channel. Within the cell, chromium (VI) is reduced to highly reactive chromium (V) and (IV), which catalyze the formation of reactive oxygen species resulting in DNA damage. In contrast, chromium (III) compounds are unable to enter cells and are not associated with carcinogenicity.

Nonessential Metals

Many metal compounds of higher order in the periodic system with a specific density above 5 ("heavy metals") have in common that they either tend to interact with amines, sulfhydryls, or carboxylic groups of biological macromolecules or possess a catalytic activity, thus deteriorating biological functions (Rajkumar and Gupta 2020). Beyond such general modes of action, each metal compound has its own characteristic toxicity pattern. Here follow some examples:

Metal toxicity can only occur when the metal (or metal-containing compound) is bioavailable. For example, **barium sulfate is** used in gastrointestinal X-ray diagnosis as contrast medium. It is highly insoluble and virtually nonabsorbable in the intestine. In contrast, soluble barium compounds are readily absorbed, cause hypokalemia, and are associated with cardiac toxicity.

Cadmium sulfide is virtually insoluble in water and not much absorbed after oral intake, whereas soluble cadmium species or inhaled cadmium compounds are toxic causing acute liver damage and chronic kidney disease and are classified as carcinogens. Tobacco smoking is a relevant source for cadmium intake. Cadmium at elevated levels is in part bound to the metal-binding protein metallothionein.

Lead inhibits the enzyme aminolevulinic acid dehydratase and thus depresses heme synthesis. Moreover, it is very neurotoxic and has various additional targets of toxicity. It tends to accumulate in the organism. The organic derivative tetraethyl lead, formerly used in large amounts as antiknock agent for petrol to improve engine performance, is a volatile liquid that is readily absorbed via the inhalative and dermal routes. This substance is primarily neurotoxic.

Mercury had in the past many technical and medical applications but was gradually abandoned and banned in most items due to its chronic toxicity that varies with its chemical form. Metallic mercury is volatile; when inhaled, it is sufficiently lipophilic to get absorbed and to cross the blood-brain barrier, where it gets oxidized by enzymes and retained in the brain for years, causing neurotoxicity. Mercury salts have adverse effects on various organs, depending on the type of salt. Methylmercury is neurotoxic and embryotoxic.

Thallium intoxications, when survived, are characterized by the loss of hair.

Osmium tetroxide is a volatile, lipid soluble liquid. It interacts with various types of biomolecules, such as double bonds of phospholipids. When airborne, it causes damage of the eye and airways. It is considered to be the most toxic metal compound.

This section so far has shown that each metal has its individual type of toxicity. Nevertheless, there are some common aspects relevant to the concept of intrinsic toxicity:

- Elevated exposures to metals and their compounds may constitute a health risk.
- Metals can be analyzed by atomic absorption spectrometry.

- Patients with metal intoxication receive metal-chelating agents as treatment to enforce elimination.
- Once in the environment, metal atoms will persist.

To protect humans from toxic metal exposures, numerous national and international recommendations, guidelines, and limit values have been defined, for virtually all regulated areas, including food, drinking water, toys, cosmetics, pharmaceuticals, soils, and sewage sludge; it would be beyond the scope of this chapter to give references. At the same time, new semiconductors and catalysts bring new types of metals (e.g., platinum-group metals as catalysts) into the environment, for which the long-term toxic consequences are not yet clear.

Target-Related Toxicity

Prologue: Damage and Repair

An understanding of the mode of action of a toxic substance is of scientific interest but has also practical regulatory consequences, such as decisions to ban or tightly regulate a chemical or group of chemically related substances that pose a high risk.

Some toxicants, e.g., the organophosphates, deteriorate just a single type of enzyme, thus causing dysfunction and sometimes death of the organism. Other toxicants are universally reactive chemicals (e.g., mustards) that form covalent bonds with many types of cellular molecules and thus cause cell death in the exposed tissue.

There exist two types of cytotoxicity-induced cell death: When ATP synthesis and basic biochemical pathways of a severely damaged cell are still functioning, the cell initiates programmed cell death (apoptosis) (Elmore 2007). One of the apoptosis pathways is triggered in a multistep pathway by release of cytochrome c from the mitochondrial membrane. In the process of apoptosis, the cell starts degrading its contents in an ordered manner and releases local mediators that attract neighboring cells to migrate toward the injured area. Proliferation of nearby organ-specific stem cells finally replaces lost cells by newly formed daughter cells (not in all tissues).

In contrast, massive cytotoxic damage rapidly leads to unordered lysis of cells (necrosis) and release of debris. Tissue damage after necrosis tends to be more complex than after apoptosis, involving inflammation to remove the cell debris.

Depending on the severity of damage and type of toxic injury, repair may ultimately result in full tissue regeneration or defective healing. Defective healing involves inflammation, whereby immune cells degrade cell debris. They also send out signal substances that may attract fibroblasts. This may lead to an irreversible replacement of functional organ cells by connective tissue. Repair velocities vary among tissues. Skin and mucous membranes have a rapid tissue repair capacity, but neuronal cells only a very restricted ability for cell recovery or renewal. Knowledge of such pathobiological processes is important for understanding tissue alterations, induced by either single acute exposure or repeated chronic lower-dose exposures.

Molecular and Organ Level

Intrinsic toxicity can be seen not only from the viewpoint of the toxic agent (as shown above) but also from the viewpoint of organ-specific adversities.

Membrane Potential and Neuronal Activity

Resting Membrane Potential

The resting membrane potential of cells is maintained by the sodium/potassium ATPase activity in the cell membrane that shuffles potassium ions from the extracellular space into the cell in exchange to sodium ions. However, potassium ions tend to leak back into the extracellular space, and the concomitant outward-directed positive charge is the origin of the cell's resting membrane potential. The sodium/potassium pump is inhibited by the digitalis poisons of the foxglove. Intoxication leads to nausea, vomiting, and multiple other CNS effects. The heart muscle is particularly sensitive. Depending on other factors such as plasma-potassium and noradrenalin, higher doses of ouabain, digitoxin, etc., cause potentially fatal cardiac arrhythmias.

Gramicidin integrates into biological membranes as a transmembrane pore that allows small cations to cross the membrane uncontrolled; valinomycin is an ionophore that shuttles potassium ions from one side of the membrane to the other. These are just examples for adverse interactions that cause a collapse of the membrane potential.

Nerve Conductance

Nerve systems contain a variety of different cell types. Among them, the nerve cells (neurons) play the key role, since they are electrically excitable and can conduct electrical impulses. Their cell bodies have dendrites and long axons that extent to other nerve cells, sensory neurons, and the musculoskeletal system. Neuronal information is propagated via an electric pulse (action potential) along the membrane of the axons (neuronal conduction) and via chemical transmission (neurotransmission) across the junctions (synapses) between communicating neurons. Acute neurotoxicity is usually due to stimulation or inhibition of either mechanism.

Action potential: The action potential is generated by voltage-gated ion channels in the membrane of nerve cells, notably for sodium, that are opened when an excitation or an action potential impulse arrives, thus depolarizing the membrane and propagating the impulse. Thereafter, the channels close rapidly.

Here are some examples for adverse reactions: Tetrodotoxin, the poison of the puffer fish, as well as saxitoxin, a substance that is responsible for paralytic shellfish poisoning, are blockers of the voltage gated sodium channel, resulting in an inability to propagate an action potential.

In contrast, aconitine, the alkaloid of the plant monkshood, keeps the voltage-gated sodium channel open, even after termination of an action potential, inducing a domino effect on potassium and calcium fluxes that may result in release of

acetylcholine into the synaptic space. Pyrethroids also keep the channel in the open state, thus paralyzing insects.

Cardiac Long QT Syndrome

Cardiac action potentials are not initiated (though affected) by nerve activity but initiated by the autonomous cardiac pacemaker. The impulse is started and propagated by myocardial cells, which constitute the electrical conduction system of the heart. Deviations from "normal" can be diagnosed in the electrocardiogram. Various therapeutic drugs affect the ion fluxes by interaction with sodium and potassium ion channels. This causes a change of conductivity, visible as long QT syndrome in the electrocardiogram (ECG), thus increasing the risk of cardiac arrhythmias (Lester et al. 2019). It is therefore generally recommended to analyze such risks early during drug development or, when prescribing such medications, to control the patient's ECG.

Neurotransmission

Neurotransmission across chemical synapses can be adversely affected by many substances that increase or decrease neurotransmission. Adverse (activating or inhibiting) interactions with neurotransmission of acetylcholine (ACh), for example, include these mechanisms:

1. ACh release into the synaptic space (e.g., BoNT inhibits).
2. Binding to the postsynaptic ACh receptor (e.g., neonicotinoids activate, whereas atropine inhibits).
3. ACh degradation (e.g., organophosphates inhibit).
4. Choline reuptake (e.g., hemicholine inhibits).

Since neurotransmitter receptors exist in various subtypes that are heterogeneously distributed within the functional areas of the central nervous system, each substance that affects that network tends to exhibit its peculiar pattern of psychotoxic and neurotoxic symptoms.

Additional mechanisms of neurotoxicity exist. For example, a case of contamination of the synthetic opioid-mimetic MPPP ("1-methyl-4-phenyl-4-propionoxypiperidine") with MPTP ("1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine") has been described. The latter is converted in the brain by monoamine oxidase to the neurotoxic agent MPP^+ and induces Parkinson's disease (Langston 2017).

Moreover, many acutely neurotoxic agents are chronically neurodegenerative; ethanol is a simple example. In conclusion, substances belonging to different chemical classes may cause adverse effects on neuronal transmission.

Reproductive and Developmental Effects

Reproductive toxicology deals with genotoxic effects and chromosomal aberration in germ cells as well as non-genotoxic adverse effects on viability and count of sperm cells. *Developmental toxicology* deals with tissue development and differentiation of the embryo/fetus and disturbances by toxic and teratogenic substances. For the

safety of the pregnant women and the developing child, protective regulations are essential. Here we enumerate some of the challenges associated with the detection of adversely acting substances.

First, teratogens such as thalidomide adversely interact with specific tissue differentiation regulating morphogens that are often active only during a very short time period, sometimes just one day.

Second, the developing brain is very susceptible to damage by neurotoxic agents (e.g., ethanol, methyl mercury, neuroactive drugs) transferred from the mother to the child; intellectual deficits may become evident at birth or later in childhood.

Third, an adverse effect to the embryo may arise indirectly from substances that affect hormones or the placental sufficiency of the pregnant woman.

Fourth, oocytes of females are formed already in the embryo. Each egg then remains in an arrest state until its ovulation. This poses a risk of cumulative genotoxic damage, while at the same time oocytes have less DNA-repair capacity compared to diploid cells. The two-generation test aims at detecting adverse effects that become visible in the second generation (see also OECD testing guidelines 414, 415, 416, and 443 (OECD 2020) and ICH test guideline S5 (ICH 2020)).

Irritation

Irritation is a disorder, usually seen on skin, conjunctivae of the eyes, and mucous membranes of the respiratory tract and the gastrointestinal tract, disappearing soon after termination of exposure. Nerve endings carry sensor biomolecules (TRPV, transient receptor potential vanilloid) capable of binding many types of irritating substances; capsaicin is a prototype. Ligand binding initiates a local neurogenic inflammation, including a pain signal to the brain and a signal for local attraction of white blood cells, thus causing inflammation at the contact site. Tear gas acts via the TRPV receptors in irritating exposed epithelia of eyes and upper airways.

Low levels of a corrosive substance may cause a simple irritation, while higher doses are corrosive. Chronic tissue irritation can have further-reaching consequences, including the long-term formation of neoplasms.

Sensitization

Sensitization results from priming exposures that activate the immune system to become responsive and reactive to subsequent exposures, which then cause an allergy. The mechanism involves binding of the substance to biomolecules, detection of “foreign” epitopes by specialized cells of the immune system, and expansion of lymphocyte clones that produce corresponding antibodies or carry antibody-related molecules on their surface directed against the foreign molecule. After an initiating sensitization processes, the immune system is triggered to fully develop an allergic reaction when subsequent exposures to the substance occur.

In daily life, sensitizations are often induced by proteins. However, chemically reactive small molecule substances may also become sensitizers when binding cellular proteins; the substance-protein complex then may constitute an epitope that is recognized by the immune system as “foreign.”

Sensitization tests in vivo like the local lymph node assay (OECD 2010) and in vitro skin sensitization tests (OECD 2018) can detect early immunological and cellular response parameters that are associated with sensitization. Regulations in food safety are aimed at banning known allergic materials for use as food additives. However, for persons who have an allergy to a component of natural food, e.g., of nuts, fish, milk, or the wheat ingredient gluten, a clear labeling and declaration of the ingredients of food products is a necessary and reasonable regulatory solution that helps affected individuals to avoid the allergen.

Genotoxicity, Mutagenicity, Carcinogenicity

Genotoxicity is the feature of a substance to produce changes in DNA, such as DNA strand breaks, adducts, and frameshifts, as well as chromosomal aberration and breakage. Genotoxicity can be studied in vivo by investigating chromosomal changes (clastogenicity, aneuploidy), DNA fragmentation or nucleotide adducts, or in vitro, like in the bacterial mutagenicity tests. The latter is usually performed in the presence of a liver microsomal system to convert the test substance to its phase I metabolites, including any reactive intermediate with genotoxic/mutagenic properties (see respective OECD test guidelines for chemicals (OECD 2020) and ICH test guidelines for pharmaceuticals (ICH 2020)).

Depending on severity and location within the DNA strand, genotoxic effects may cause acute cell death (necrosis or apoptosis), or the cell may survive and repair the damage or survive with a mutation. In the latter case, the final outcome will depend on the type of the affected cell:

(a) Fully differentiated organ cell: The damage tends to remain locally restricted.
(b) Organ stem cell: The mutation may proliferate to daughter cells within the organ/tissue. Chemically induced cancer develops as a process that starts after an initial genotoxic DNA damage (initiation) in an organ stem cell and proceeds stepwise via promotion and progression.
(c) Germ cell: The mutation can get proliferated to the next generation.

Genotoxic carcinogens are thought to have no zero risk threshold. But non-genotoxic tumor-promoting agents like some hormones (e.g., estrogens) and endocrine disruptors (Del Pup et al. 2016) are thought to have a threshold, below which there is no risk.

Since carcinogenesis is a complex multistep biomolecular process that develops over time, affected by hereditary – and many other influences, the proof for an intrinsic carcinogenicity of a substance usually requires long-term (two years) studies in animals. When interpreting data of animal carcinogenicity studies, one should be aware that high doses of a substance are often applied, sometimes in a subtoxic dose range, which might induce cytotoxicity-related changes and tumor promotion. A full pattern of the toxicokinetics helps to interpret mechanisms and results. When animal experiments don‘t provide clear dose-response relationships, notably in the low-dose range, extrapolation methods help to define a probable risk level.

Epidemiological findings are very valuable when there is a stable association between exposure levels and the incidence of a human tumor type. But solid information is available only for a restricted number of mainly "old" substances. Epidemiological findings are often equivocal, not least because of mixed exposures, varying exposure levels, and other influencing factors.

Liver as Target Organ

The liver is the body's "sewage plant" for endogenous catabolic products and xenobiotics. Following intestinal absorption, it takes up nutrients and xenobiotics from the portal blood (hepatic first pass clearance), or from the circulation; modifies the substances chemically, to detoxify them; and releases the products both back to the blood and via membrane transporters (exporters) of the canalicular membrane into primary bile. Depending on the type of intrinsic toxicity, the liver may respond with different kinds of injury. Here follow some examples.

Intrahepatic Cholestasis

Intrahepatic cholestasis is characterized by reduced bile flow, often induced by pharmaceuticals, which either inhibit exporters or deteriorate bile flow by lowering bile fluidity or inducing an inflammation that compresses bile canaliculi. Reduced bile formation and bile flow result in elevated levels of xenobiotics and bilirubin in the blood (Sundaram and Bjornsson 2017).

Drug-Induced Autoimmune Hepatitis

This rare event may be induced by some drugs such as nitrofurantoin or halothane, which in the course of their hepatic metabolism form neo-antigens, probably due to protein-binding of their reactive intermediates. Upon repeated exposure, a then sensitized immune system may handle such adducts like (auto)antigens, potentially causing liver cell necrosis and inflammatory infiltration.

Liver Cell Necrosis

This type of hepatotoxicity is often the result of cytochrome P450-dependent biotransformation of xenobiotics that generates reactive intermediates, which then cause damage to liver cells. Examples are carbon tetrachloride, dimethyl nitrosamine, and aflatoxin. At high acute doses, they cause acute liver cell necrosis; chronically they are associated with the risk of liver cirrhosis and liver cancer.

Acetaminophen is a safe analgesic, when used according to prescription. A therapeutic dose is conjugated with glucuronic acid and sulfate; only a small portion is metabolized by cytochrome P450 enzymes that produce reactive intermediates, which normally are readily detoxified by conjugation with glutathione. However, excessive overdosing saturates the glucuronidation and sulfation pathways. The surplus of reactive intermediates then depletes hepatocellular glutathione (a major cellular antioxidant) and binds to proteins, thus causing liver cell death, sometimes

with fatal outcome (McGill et al. 2012). Overdosing acetaminophen is a major cause of drug-induced liver failure in the USA and Great Britain.

The poisonous mushroom "death cap" contains amanitin and several related cyclic oligopeptides that together are named amatoxin. These heat-resistant peptides are absorbed in the intestine and accumulated in liver cells, where they inhibit the enzyme RNA polymerase II and thus hepatic protein synthesis. The continuing loss of vital cellular proteins results in liver cell necrosis and often death after about two days. The other toxin of the "death cap," phalloidin, is poorly absorbed, but when injected, it is taken up by liver cells, associates with F-actin, and thus inhibits the dynamics of the cytoskeleton.

Fatty Liver

Ethanol is a major causative factor for fatty liver. Ingested ethanol gets oxidized in the liver (and other organs) to acetaldehyde and subsequently to acetic acid, which is converted to acetyl-CoA. The oxidation reactions deliver NADH/NADPH. The reaction products are substrates/cosubstrates for triglyceride synthesis. Fat droplets may transiently get stored in liver cells, thus expanding liver volume, initially without symptoms, but chronically often associated with lipid disorders and a risk for progressive liver disease.

Liver Cirrhosis

Liver cirrhosis develops as a long-term response to severe or repeated hepatotoxic exposures (e.g., ethanol, carbon tetrachloride) and other causes. It is characterized by a shrinking liver volume, replacement of liver cells by connective tissue, and disturbed liver architecture.

Liver Cancer (Hepatocellular Carcinoma)

Primary liver cancer may develop as a consequence either of massive short-time exposure or of chronic low-level exposure to substances that are genotoxic or converted to genotoxic metabolites in liver cells, such as aflatoxin or nitrosamines. Tumor promoters such as ethanol, chlorinated aromatic hydrocarbons, and viral infections tend to speed up the appearance of a tumor.

Hepatic Encephalopathy

A partly damaged liver has a reduced capacity to detoxify catabolic products and xenobiotics. This leads to accumulation of neurotoxic substances such as ammonia as well as metabolic products of intestinal microbiota that are insufficiently eliminated by a damaged liver, thus exerting narcotic effects.

Role of Individual Susceptibility

Intrinsic toxicity is the potential of a substance to produce an adverse effect. However, whether a toxic effect occurs may depend on additional conditions, such as individual sensitivities. Regulations covering an entire population just to protect

few susceptible individuals are sometimes not feasible. In such cases, regulations may provide recommendations and information that help the susceptible individuals to avoid the adverse exposures (e.g., pharmaceuticals package insert).

NSAID-Induced Ulcer Disease

Nonsteroidal anti-inflammatory drugs (NSAID) are used for pain control, often chronically. Many individuals tolerate recommended doses without gastrointestinal problems. But some individuals, notably the elderly and those under stress, may develop a gastroduodenal ulcer (Hunt and Yuan 2011). This is in part due to the intrinsic inhibitory effect of NSAIDs on prostaglandin synthesis, whereby the gastro-protecting effects of prostaglandins are decreased (e.g., blood flow, mucous secretion, bicarbonate secretion).

Asbestos, Cigarette Smoke, and Lung Cancer

Asbestos exposure is a risk for lung cancer and for mesothelioma. Smoking is a high risk for lung cancer. Asbestos workers who smoke have an explicitly potentiated risk to develop lung cancer compared to nonsmoking asbestos workers (Klebe et al. 2019).

Gluten and Celiac Disease

Celiac disease is an inflammatory reaction of the intestinal mucosa triggered by gluten of wheat and structurally related proteins of other cereals (Lerner et al. 2017). It involves the following mechanisms: Gliadin molecules are poor substrates for gastric and intestinal proteases. When the intestinal barrier is compromised, they can gain access to the subepithelial tissue, where they get deaminated by tissue transglutaminase. As side product of this catalytic process, protein adducts may be formed. These are identified by the adaptive immune system of predisposed individuals as "foreign" neo-antigens, inducing sensitization, local inflammation, and an autoimmune response with production of antibodies. When exposure continues after the initial sensitization, the now inflamed mucosa loses its barrier function as well as the ability to efficiently absorb nutrients. Gluten is categorized as food allergen. An essential help for individuals with celiac disease is the labeling of gluten-containing and/or gluten-free food products.

Alpha-1 Antitrypsin Deficiency and Lung Function

Alpha-1 antitrypsin is an endogenous inhibitor of overshooting, tissue damaging protease activity, notably in the lung. The pulmonary form of the genetic disorder

"alpha-1 antitrypsin deficiency" is associated with accelerated aging of the lung (emphysema) and airway hypersensitivity toward inhaled irritating chemicals and smoke.

Iron and Iron Storage Disease

In hereditary hemochromatosis, severalfold more iron tends to be absorbed from food than in normal conditions. The iron overload causes liver disease and other disorders. This reveals the intrinsic toxicity of iron, when the normal tight biological regulation of iron levels fails.

Age-Dependent Sensitivities

Fetus: Ethanol readily distributes across the blood-placenta barrier, so that the developing fetus and its neuronal system are exposed to ethanol in drinking pregnant women, sometimes with the outcome of an irreversible fetal alcohol syndrome of the offspring, characterized by deficits in physical and mental capabilities.

Newborns: Opiates and opioids taken by pregnant women may cause acute respiratory depression and withdrawal syndromes after delivery.

Children: The metal lead and its compounds are more toxic in children compared to adults, because children absorb the metal more efficiently and their developing brain is more susceptible to the intrinsic neurotoxicity of lead compared to adults.

Elderly people: Organ function declines with age. This is evident with heart function, lung function, liver function, and kidney function and associated changes of blood circulation. So elderly people are more susceptible to substances with the intrinsic toxic feature of inhibiting oxygen transport by hemoglobin (e.g., carbon monoxide) or oxygen utilization for ATP production in mitochondria (cyanide). As renal glomerular filtration and hepatic drug-metabolizing capacities decrease with age, dosing of many pharmaceutical drugs must be adapted to avoid overdosing.

Poor Metabolizers

Due to the polymorphism of cytochrome P450 isoenzymes, the metabolic rate of some substances varies considerably (Leeder 2015) even among healthy young individuals. Poor (slow) metabolism of a substance may be associated with excessive or prolonged effects of pharmaceuticals. An example is the unusually strong decline of blood pressure after repeated intake of the antihypertensive drug debrisoquine that is metabolized by cytochrome P450 2D6. Slowly metabolizing individuals tend to accumulate various drugs (e.g., amitriptyline) with the risk of overdosing and side effects. Genetic deficiency of plasma cholinesterase causes delayed metabolism of the muscle relaxant succinylcholine that is used in anesthesia.

Summary

Knowledge of intrinsic toxicity concepts may support the risk manager to anticipate the hazard of a chemical for which no toxic profile is known. Such knowledge helps the clinical toxicologist who is confronted with certain symptoms of an intoxicated patient, to make assumptions about the substance that produces such symptoms. The intrinsic toxicity concept is the basis for the analogy principles (chemical-structure analogy and biological effect/symptom analogy) and for bridging approaches that are used to estimate toxicities in situations where toxicological information is incomplete or missing.

Cross-References

- ▶ Do Carcinogens Have a Threshold Dose? The Pros and Cons
- ▶ Importance of Physicochemical and Physical Properties for Toxicological Risk Assessment
- ▶ Nanoparticles and Their Regulation
- ▶ Psychoactive Designer Drugs: Classes, Mechanisms, and Regulation
- ▶ Read-Across Methodology in Toxicological Risk Assessment
- ▶ Susceptible Population Groups in Regulatory Toxicology
- ▶ Toxicodynamic Tests
- ▶ Toxicokinetic Models

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Importance of Xenobiotic Metabolism: Mechanistic Considerations Relevant for Regulation

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Franz Oesch and Jan G. Hengstler

Contents

Abstract

Most xenobiotic compounds which require regulation undergo metabolic alterations in the human organism which frequently differ from those occurring in nonhuman species and in in vitro experimental test systems. Some of these differences are fundamental, including the complete absence of metabolic steps which are crucial for the toxicity of the compound under consideration. Examples of crucial species differences in toxicity-related xenobiotic metabolism include MeIQx (2-amino-3,8-dimethylimadazo[4,5-f]quinoxaline), aflatoxin B1, and vinyl acetate which are discussed in this chapter.

Keywords

Adduct · Carcinogen · Cytochrome P450 · Biotransformation · Genotoxicity Assay · Metabolic activation · Safety Assessment · Toxication · Xenobiotic

F. Oesch (✉)
Institute of Toxicology, Johannes Gutenberg-University, Mainz, Germany
e-mail: oesch@uni-mainz.de

J. G. Hengstler
Leibniz Research Centre for Working Environment and Human Factors at the Technical University of Dortmund (IfADo), Dortmund, Germany
e-mail: hengstler@ifado.de

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Fundamentals

Most xenobiotics do not remain unchanged in the human organism (and in other organisms), but rather are metabolized (Oesch-Bartlomowicz and Oesch 2007). The change in chemical characteristics of the thereby produced metabolites as compared with the chemical characteristics of the corresponding parent compounds usually leads to changes in both, the desired properties, for example, therapeutic efficacy, and also in undesired properties, that is, xenobiotic metabolism usually is toxicologically not neutral, but rather leads in most cases to toxication or detoxication of the respective compound (for an overview see Oesch-Bartlomowicz and Oesch 2007). Thereby xenobiotic metabolism becomes one of the most important factors controlling the toxicity of the respective compound. This, in turn, makes xenobiotic metabolizing enzymes to control factors for xenobiotic toxicity. These xenobiotic metabolizing enzymes drastically differ quantitatively and in many instances even qualitatively between animal species, organs, cell types, developmental stages, and physiological states such as health and individual diseases, most often even between strains and genders (for a succinct review see Hengstler and Oesch 1999). For an extrapolation of toxicity findings in experimental systems to humans, cognizance of differences in xenobiotic metabolizing enzymes between the systems used and humans is therefore critical.

Quantity and also chemical identity of the formed metabolites depend on many factors. This complexity leads to difficulties to predict from experimental systems which metabolites and how much of them will be generated in humans. Difference in xenobiotic metabolism between species is one of the factors which most profoundly limit the extrapolation of toxicological results obtained in experimental systems to humans (for a comprehensive review see Hengstler et al. 1999). The later considerations in this chapter will therefore especially take this interspecies parameter into account. However, in order to be comprehensive, it must also be stated that in addition to these crucial species differences, further important differences exist also within a given species. This includes genetic differences in xenobiotic metabolism between strains and between individuals. In humans genetic polymorphisms in xenobiotic metabolizing enzymes known to-date account for up to 40% of cytochrome P450 (CYP) dependent xenobiotic metabolism (Modak 2010). The generally used pharmaco−/toxicokinetic default uncertainty factor of 3.2 to account for human interindividual differences in the extrapolation of toxicity data to human may need to be enlarged if it does not encompass human polymorphisms from poor to extensive metabolizers of the xenobiotic compound in question (Schroeder et al. 2011). Moreover, differences caused by different gene expressions during development and disease states drastically influence xenobiotic metabolism, most profoundly if the liver, the organ responsible for the largest portion of the mammalian xenobiotic metabolism, is involved. In addition, environmental factors, nutrition, and drug treatment can profoundly modulate xenobiotic metabolism by enzyme induction or repression, activation or inhibition (for an overview see Oesch and Arand 1999; updated version in German: Arand and Oesch 2019). These numerous factors may interact with each other, generating a high complexity of xenobiotic metabolism

control and consequent toxicities. For instance, very early on, it had already been shown that differences in nutritional status profoundly influenced drug metabolizing enzymes induction (e.g., by DDT) and the consequent toxicity of a third compound (e.g., carbon tetrachloride) (McLean and McLean 1966).

In order to improve the water solubility and excretability of xenobiotics, the organism most often uses conjugation with endogenous water-soluble building blocks such as glutathione, glucuronic acid, or sulfate. Such conjugations need the preexistence of suitable substituents in the xenobiotic compound in question which, if not preexisting in the parent compound, first have to be introduced or liberated. This step in xenobiotic metabolism is called phase I, and the subsequent conjugation is called phase II (the then frequently following active excretion of the generated water soluble metabolite from the cell of origin is often called phase III).

The Phase I metabolites possess at the site to be conjugated electrophilic (such as epoxides, α,β-unsaturated carbonyls) or nucleophilic (such as hydroxyl, sulfhydryl, amino, carboxyl) structural components. Depending on their relative chemical reactivities, electrophilic moieties can have high toxicological potential by reacting with nucleophilic moieties of endogenous compounds. This is toxicologically most significant if the electrophilic moieties modify the structures of macromolecules such as proteins, RNA, and – especially important – DNA. The latter potentially leads to significant genotoxicity. In contrast to this, nucleophilic metabolites usually do not covalently react with endogenous molecules and therefore usually are toxicologically less problematic. However, they can, in some cases, have affinity to receptors and thereby lead to desired therapeutic or undesired toxic interactions.

The conjugating phase II reactions in most cases lead to a large increase in the water solubility of the compound in question, to its efficient excretion and to termination of its biological activity, be it beneficial (therapeutic) or undesired (toxic). However, some important exceptions exist. For instance, some glucuronides (e.g., of morphine) possess high biological activities; some conjugates (e.g., of vicinal halogenated alkanes) with glutathione possess higher genotoxic potential then the parent compound (for an overview see Oesch-Bartlomowicz and Oesch 2007).

The enzymes catalyzing phase I reactions include oxidoreductases and hydrolases. Oxidoreductases relevant for xenobiotic metabolism include cytochromes P450 (CYP), flavin-containing monooxygenases (FMO), monoamine oxidases (MAO), and cyclooxygenases (COX). In most cases these oxidoreductases introduce oxygen into xenobiotic molecules or abstract hydrogen or electrons. CYPs are quantitatively especially often involved in xenobiotic metabolism. Thus, two-thirds of the top 200 drugs prescribed in the United States (year of survey: 2002) are cleared through metabolism that involves CYPs (Williams et al. 2004). Further important xenobiotic metabolizing oxidoreductases include dehydrogenases and reductases such as alcohol dehydrogenases (ADH), aldehyde dehydrogenases (ALDH), and carbonyl reductases. They abstract or add hydrogen atoms. Diverse xenobiotic metabolizing hydrolases catalyze the hydrolysis of esters, amides, glucuronides, sulfates, or epoxides.

In the phases II reactions electrophilic substrates are conjugated by glutathione S-transferases (GST), nucleophilic substrates by UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), N-acetyltransferases (NAT), acyl-CoA-aminoacid-N-acyl-transferases, and methyltransferases (for an overview see Oesch and Arand 1999; updated version in German: Arand and Oesch 2019).

A correct prediction of toxicity is especially important in cases of long latencies such as cancer, since a wrong prediction leads to accumulation of numerous irreversible damages before the error becomes manifest. For such toxicities, electrophilically reactive metabolites are especially important which frequently have a short life span and are formed in low quantities. For such cases, cognizance of reactive metabolites and xenobiotic metabolizing enzymes responsible for their control (formation, detoxication, sequestration into alternative pathways) is especially important. Examples of some important electrophilically reactive metabolites and xenobiotic metabolizing enzymes involved in their control are given in Table 1. An important consequence of the fact that quantitatively minor metabolites may be responsible for toxic (especially for genotoxic) effects is that species-specific divergent pathways leading to such minor but toxicologically important metabolites may become crucial. When a human-only metabolite is not formed in the experimental species chosen for toxicity testing, an incomplete xenobiotic safety assessment may result leading to an underestimation of toxicological risk. The FDA/CDER guidance on safety testing of drug metabolites (U.S. Department of Health and Human Services 2008/2009) therefore states that a unique human metabolite must itself be tested for toxicity when the metabolite level reaches >10% parent systemic exposure at steady state.

Some overall approximations in relatively high similarities of some xenobiotic metabolizing enzymes or their response to exogenous stimuli between certain experimental animal species and humans may be attempted. Although the different animal models have many differences in the ligand-binding domain of the

Table 1 Reactive metabolites: Some important prototypes

Parent compounds	Reactive metabolites	Enzymes involved in the control
Aromatic/olefinic	Epoxides	Cytochromes P450
hydrocarbons		Glutathione S-transferases
		Epoxide hydrolases
Aromatic/heterocyclic amines	Reactive esters	Cytochromes P450
		Sulfotransferases
		Acetyltransferases
		Glutathione S-transferases
		UDP-glucuronosyltransferases
Dialkylnitrosamines	Carbonium ions	Cytochromes P450
	Electron deficient alkyl groups	
Vicinal dihaloalkanes	Episulfonium ions	Glutathione S-transferases

respective nuclear receptors involved in the control of xenobiotic metabolizing enzymes induction compared with humans (Mohutsky et al. 2010; Evans and Mangelsdorf 2014), induction responses compared with humans appear to be most similar in rats and mice for CYP1A; in rats, mice, and pigs for CYP3A; in monkeys for CYP2C; and in dogs for CYP2D (Martignoni et al. 2006; Zuber et al. 2002; Bogaards et al. 2000). However, some exceptions of outstanding practical importance highlight the fact that a priori acceptance of these overall relatively high similarities may be dramatically misleading for an individual xenobiotic compound under consideration. Thus, rifampicin does not induce CYP3A in rats or mice, but does so in humans (leading to unwanted pregnancies in combined use of contraceptives and rifampicin) and in rabbits (Kocarek et al. 1995; Back et al. 1988). Inversely, pregnenolone-16α-carbonitrile (PCN), which strongly induces CYP3A in rats and mice, causes no induction in humans or rabbits, and CYP3A induction by 5α-pregnane-3,20-dione is seen only in humans and mice, but not in rats or rabbits (Mohutsky et al. 2010). For improved predictions animal models have been genetically modified in which some nuclear receptors controlling induction of a xenobiotic metabolizing enzyme of that species has been knocked out and replaced by the corresponding human gene (Ma et al. 2007: Scheer et al. 2008; Gonzalez et al. 2015).

Examples of Metabolism Associated Toxicity

Having discussed the basic aspects of drug metabolism the following chapter will focus on examples of compounds where drug metabolism plays an important role for risk assessment. Usually risk assessment is based on animal experiments. For identification of acceptable human exposures, NOAELs (see ▶ Chap. 1, "Aims and Mission of Regulatory Toxicology") from laboratory animals are used and multiplied with safety factors. Usually this procedure identifies exposure levels that are safe for humans. However, working with safety factors, for example, a fixed safety factor of 10 to consider possible interspecies differences of metabolism, may under certain circumstances lead to mistakes. This is the case when interspecies differences in metabolism between humans and the relevant animal species are huge. To illustrate this problem, some examples of well-characterized compounds will be discussed in the following paragraphs (from: Hengstler et al. 1999 and Hengstler et al. 2003 and references cited therein). It should be considered that they represent extreme and rare cases. Nevertheless, they are important to illustrate how mistakes in risk assessment can be avoided.

MeIQx (2-Amino-3,8-Dimethylimadazo[4,5-f]Quinoxaline)

MeIQx represents an intensively studied heterocyclic amine found in fried as well as cooked meat. It is formed by a heat dependent reaction between muscle creatinine and amino acids. MeIQx is a strong colon carcinogen in rats and mice. However, it

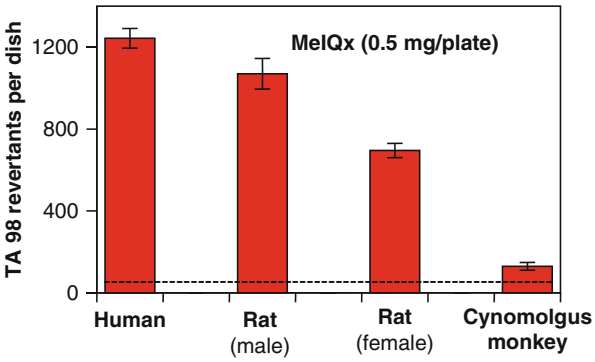

Fig. 1 Activation of the heterocyclic amine MeIQx (2-amino-3,8-dimethylimidazo-(4,5-f) quinoxaline) to a mutagenic metabolite by liver microsomes of humans, rats and cynomolgus monkeys. The dashed line shows the number of spontaneous revertants in the Ames test (Davis et al. 1993; review: Hengstler et al. 1999)

does not cause colon cancer in cynomolgus monkeys. Therefore, a critical question is whether human risk assessment should be based on the rodent or monkey data. Because of the small evolutionary distance, one might be tempted to favor the monkey for this purpose. However, a relatively simple experiment demonstrates that in this case risk assessment must be based on the more susceptible rodents. An Ames mutagenicity test using microsomes from livers of human, rat, and cynomolgus monkeys as a metabolizing system reveals major interspecies differences. Human and rat microsomes strongly activate MeIQx to a mutagen, whereas microsomes from cynomolgus monkeys are almost inactive (Fig. 1). This corresponds to the mechanism of metabolic activation of MeIQx to a carcinogenic nitrenium ion (Fig. 2). Human and rat cytochrome P450 1A2 form a hyroxylamine that is further metabolized to a reactive N-acetoxy-ester. In contrast, cynomolgus monkeys lack an activity corresponding to human or rat cytochrome P450 1A2. However, it should be considered the cynomolgus monkey represents an exception with respect to MeIQx metabolism. Even other monkey species, such as marmosets, form the hydroxyl amine from MeIQx and are therefore susceptible to its carcinogenic effect. In conclusion, humans are similarly susceptible to MeIQx induced carcinogenicity as rats and do not represent a resistant species, such as cynomolgus monkeys. Therefore, risk assessment must be based in this case on the more susceptible species.

Aflatoxin B1

Aflatoxin B1 is one of the most potent liver carcinogens for humans and rats. However, the TD50 (the dose that induces tumors in at least 50% of the animals) shows large interspecies differences, ranging between 1 and 6 μg/kg/day for different rat strains, whereas even doses of 2000 μg AFB1/kg/day did not yet cause liver tumors in 50% of the C57/BL6 mice. Therefore, the interspecies differences between rats and mice are larger than a factor of 1000, a difficult scenario for human risk assessment.

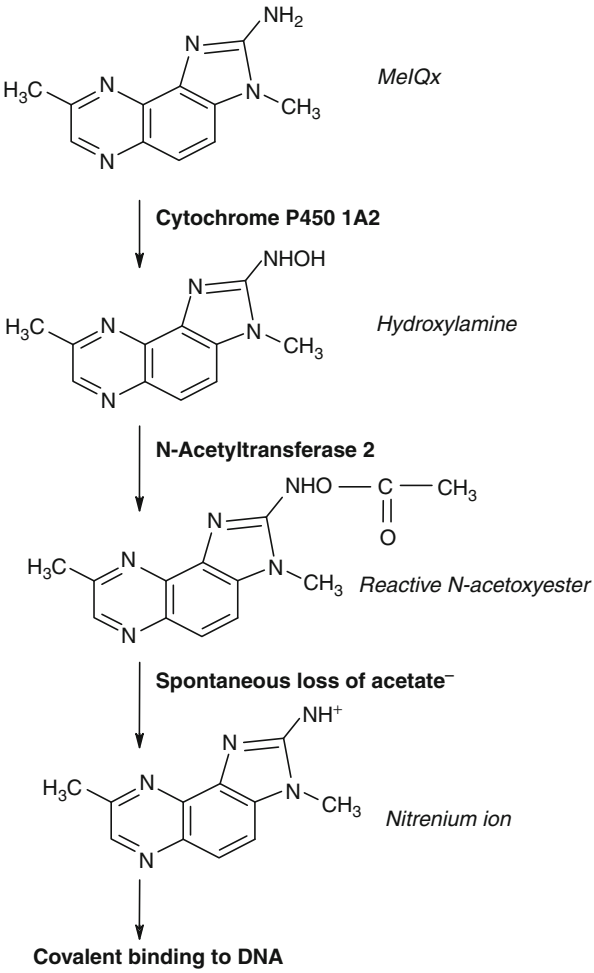

Fig. 2 Mechanism of metabolic activation of MeIQx to a DNA binding nitrenium ion by cytochrome P450 1A2 and N-acetyltransferase 2. Humans and rats efficiently form the hydroxylamine. However, cynomolgus monkeys lack a corresponding activity

To study whether humans are as susceptible to AFB1 as rats or rather as resistant as mice, genotoxicity assays were performed using liver microsomes of all three species as a metabolizing system (Fig. 3). Sister chromatide exchanges (SCE) in human lymphocytes were analyzed as a genotoxic endpoint. Incubation of AFB1 (10 μM) with liver microsomes of all three species caused a clear increase in SCEs when NADPH was added to the incubation mixture, whereby NADPH acts as a cofactor of the cytochrome P450 mediated metabolic activation of AFB1. However, metabolic activation by mouse liver microsomes was stronger compared to human and rat. It should be considered that lower AFB1 concentrations (only 1 μM for mice compared to 10 μM for human and rat) were used. This seems to be in contrast to the aforementioned carcinogenicity studies where mice appeared to be more resistant than rats. However, this discrepancy could be explained by an additional experiment

(Fig. 3). In microsomal preparations, the cofactors of phase II metabolism, such as glutathione (GSH), are too diluted to allow an in vivo like phase II metabolism. Therefore, GSH and cytosol of the corresponding species (containing, e.g., glutathione-S-transferases) were added to the microsomal incubations. These experiments showed a strongly reduced SCE induction when mouse cytosol was added (Fig. 3). In contrast, addition of cytosol and GSH did not reduce genotoxicity of human and rat microsomal incubations. Therefore, mouse liver microsomes have a higher capacity to activate AFB1 to a genotoxic species compared to humans and rats. On the other hand, the cytosolic compartment of mice also shows a higher capacity to detoxify AFB1.

Today, the mechanisms underlying these observations are known. Activation of AFB1 to a genotoxic carcinogen, namely, AFB1-exo-8,9-epoxide, is catalyzed mainly by cytochrome P450 1A2 and 3A4 (human). The extremely efficient inactivation of AFB1-exo-8,9-epoxide in mouse liver cytosol is catalyzed by the glutathione-S-transferase isoenzyme mGSTA3–3 (synonym: mGST-Yc). In contrast, humans and rats do not express phase II enzymes with a similarly high capacity to detoxify AFB1-exo-8,9-epoxide.

An adequate technique for identification of the interspecies difference in AFB1 susceptibility is analysis of DNA adducts in primary hepatocytes (Fig. 4). While an approximately similar extent of DNA adducts was formed in human and rat hepatocytes, the corresponding data of mice were below the detections limit. Finally, the glutathione conjugation capacity can directly be analyzed, illustrating the low capacity of human liver cytosol to detoxify AFB_1-8,9-epoxide compared to mice (Fig. 5). In conclusion, humans are more susceptible to AFB_1 mediated carcinogenesis than mice. Therefore, human risk assessment in this case should be based on rat data.

Vinyl Acetate – The Relevance of Practical Thresholds

The examples of MeIQx and AFB_1 have illustrated the importance of basing risk assessment on toxicity data of species that resemble the human situation. This is particularly relevant in case of huge interspecies differences of metabolic activation or detoxication. A further important aspect for risk assessment is the dose response relationship at low in vivo relevant doses. In this chapter, we discuss the example of vinyl acetate to illustrate the relevance of threshold mechanisms. Similar principles can be applied to acrylonitrile and 1,3-butadiene which also are produced in large amounts. Vinyl acetate is carcinogenic in rats and mice. After oral administration only tumors of the oral cavity, esophagus and forestomach have been observed. Inhalation studies with rats led to tumors of the olfactory epithelium. Therefore, vinyl acetate represents a typical “site of contact carcinogen.” Vinyl acetate is known to induce DNA protein adducts, chromosomal aberrations, and sister chromatid exchanges. Therefore, it represents a genotoxic carcinogen. Nevertheless, metabolism and mechanism of action of vinyl acetate show some relevant differences compared to MeIQx and AFB_1 that should be considered for risk assessment.

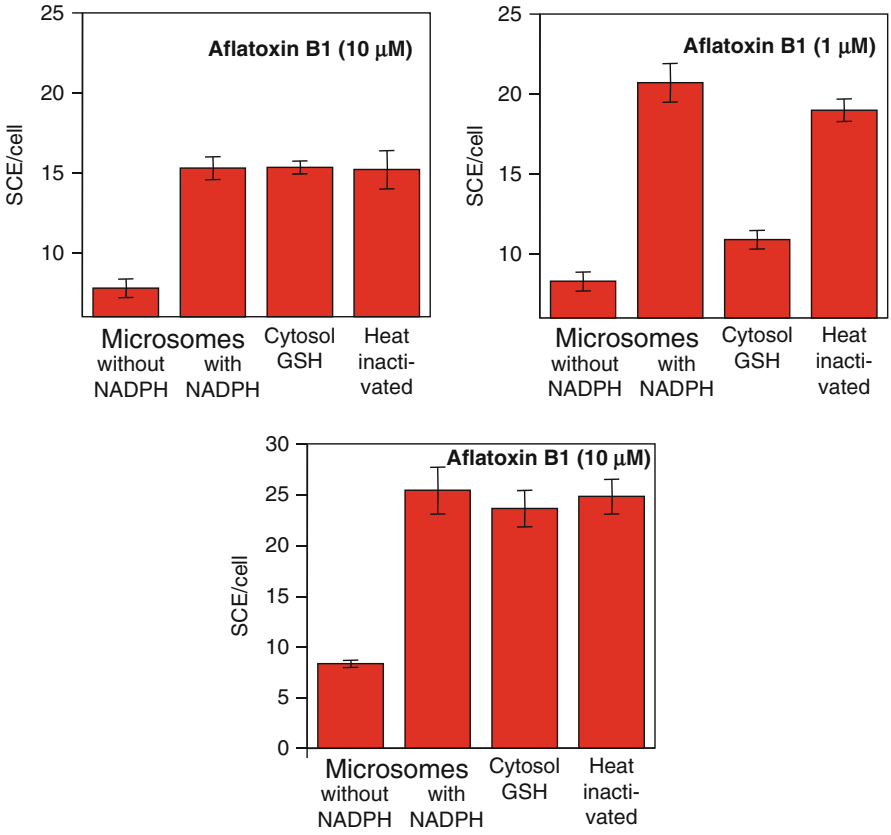

Fig. 3 Influence of phase I- and phase II-metabolism on the genotoxicity of aflatoxin B_1 (AFB1), as evidenced by the sister chromatid assay in human lymphocytes. Venous blood of a volunteer was incubated with AFB_1 in the presence of human (first chart), mouse (second chart), or rat (third chart) liver microsomes. Concentrations of 10 μM AFB_1 were used for incubations with human and rat microsomes. For incubations with mouse microsomes, only 1 μM AFB_1 was used since higher concentrations were no longer in the linear concentration-effect range. First column: negative controls with microsomes, but without the co-factor NADPH. Second column: influence of phase I metabolism in the presence of microsomes and NADPH. Third column: combined influence of phase I metabolism and glutathione-S-transferases in presence of microsomes, NADPH, cytosol, and 2.5 mM glutathione. Forth column: negative control, with the same conditions as for the third column but using heat inactivated cytosol (Wilson et al. 1997; review: Hengstler et al. 1999)

Importantly, vinyl acetate is rapidly metabolized to acetaldehyde and acetic acid. This reaction is catalyzed by carboxyl esterase and aldehyde dehydrogenase. Acetaldehyde can cause DNA-protein crosslinks and finally chromosomal aberrations at high concentrations. Acetaldehyde represents the only genotoxic metabolite of vinyl acetate. The parental compound is not genotoxic. Also, the second metabolite, acetic acid, may contribute to vinyl acetate cytotoxicity by decreasing the pH value. Decreases of the pH value of less than 0.15 units usually remain without toxic

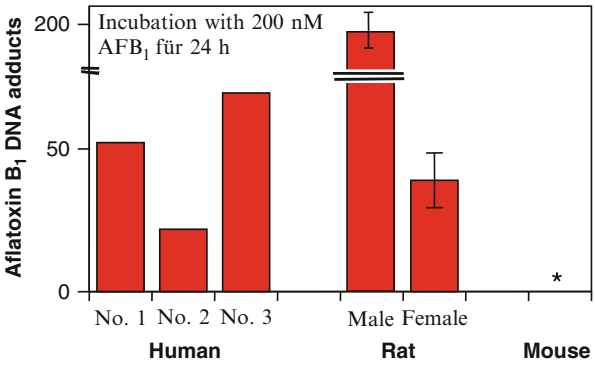

Fig. 4 DNA binding of ^{3}H-aflatoxin B_1 in cultivated hepatocytes of humans (three donors), rats (Sprague Dawley, male and female), and mice (CD-1, male) after incubation for 24 h (Cole et al. 1988; review: Hengstler et al. 1999). (*, Below detection)

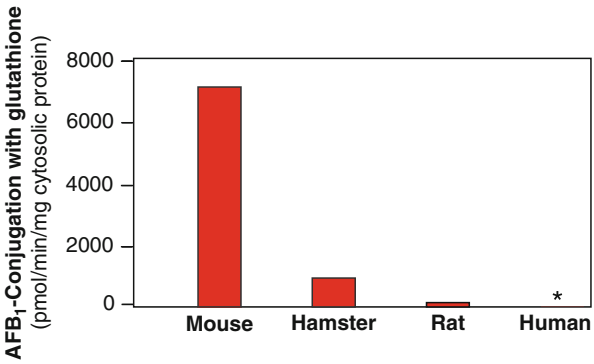

Fig. 5 Conjugation of aflatoxin B_1–8,9-epoxide (generated by incubation with microsomes) with glutathione by cytosol of livers from mice, hamsters, rats or human (Slone et al. 1995). (*Below detection limit)

consequences. However, a further decrease in pH may cause cytotoxicity and replacement proliferation. This will promote carcinogenesis resulting from acetaldehyde induced DNA lesions.

For risk assessment, it is important to consider that both metabolites of vinyl acetate, acetaldehyde, as well as acetic acid also are endogenously formed in the organism. Acetaldehyde is formed in threonine metabolism. Endogenously, acetaldehyde is present in concentrations of approximately 0.3 μg/ml blood. Exposure to vinyl acetate at levels that increase acetaldehyde and acetic acid within the endogenously occurring range does not induce tissue damage or carcinogenesis, which will be shown below. Therefore, it can be concluded that the organism has established protective mechanisms that avoid tissue damage at physiological levels of both vinyl acetate metabolites. Exposure to vinyl acetate should be acceptable if the resulting increase in acetaldehyde and acetic acid at the highest exposed cells of the organism is lower than endogenously formed concentrations.

Although the aforementioned theoretical considerations may seem plausible, the assumption of a "practical threshold" is only acceptable when proven by experimental data. Dose response experiments for vinyl acetate induced carcinogenicity

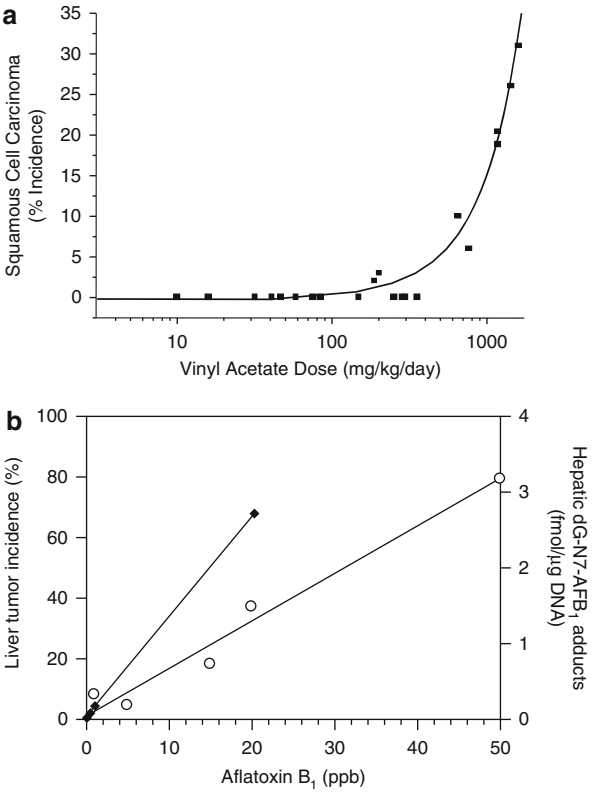

Fig. 6 (**a**) Dose response relationship between exposure to vinyl acetate in the drinking water and induction of carcinomas in rats and mice. Since rats and mice respond similarly to vinyl acetate on a mg/kg/day basis, data for rats and mice were summarized in one figure (details in Hengstler et al. 2003). Carcinomas induced by vinyl acetate under these conditions are tumors of the oral cavity, esophagus, and forestomach. The data show an increase of tumor incidence only at doses higher than 100 mg/kg/day. (**b**). Induction of liver tumors (○) and DNA-adducts (♦) by aflatoxin B_1. In contrast to vinyl acetate, a linear dose-response relationship was obtained by aflatoxin B_1 (review: Hengstler et al. 2003)

show a wide dose range without increased tumor incidence (Fig. 6a). However, a clear increase is observed at doses higher than 100 mg vinyl acetate/kg body weight/day. The shape of the dose response curve of vinyl acetate clearly differs from that of the no-threshold carcinogen AFB_1, for which both tumor incidence and DNA adducts do not show any evidence for a threshold (Fig. 6b).

The threshold of the dose-response relationship for vinyl acetate is due to the fact that critical concentrations of acetaldehyde and acetic have to accumulate up to certain concentrations where mechanisms relevant for carcinogenesis are activated. For the olfactory epithelium, five steps of vinyl acetate mediated carcinogenesis seem to be critical (Fig. 7). According to our PBPK model, exposure of 50 ppm vinyl acetate leads to acetaldehyde concentrations of 1.7 µg/ml (step 1 in Fig. 7). Moreover, the resulting acetic acid causes a pH reduction of 0.08 units in basal cells of the olfactory epithelium, the cells of origin of carcinogenesis (step 2). This pH decrease is less than the critical value of Δ pH 0.15 which may cause cytotoxicity. Therefore, degeneration of olfactory cells (step 3) and replacement proliferation (step 4), steps critical on path to cancer, do not yet occur at 50 ppm vinyl acetate. However, increasing vinyl acetate exposure to 200 or even 600 ppm will activate these

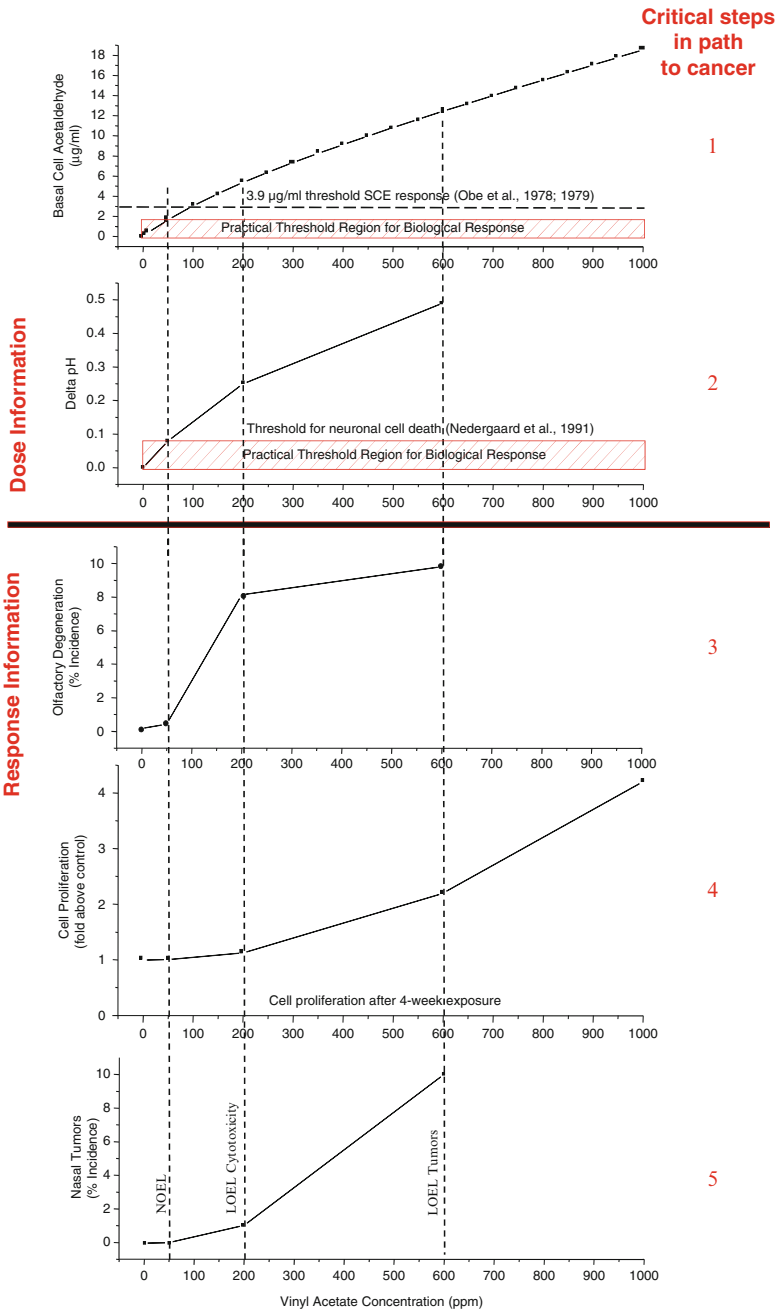

Fig. 7 Five critical steps on path to cancer induced by vinyl acetate in the olfactory epithelium (from: Hengstler et al. 2003). Panel 1: Concentrations of acetaldehyde in the basal cells of the olfactory epithelium, the cells of origin of nasal tumors. Panel 2: Reduction of pH in relation to vinyl acetate exposure. Reduced pH is responsible for cytotoxicity in the olfactory epithelium (panel 3), which causes replacement proliferation (panel 4) and finally promotes induction of nasal tumors (panel 5)

mechanisms. Vinyl acetate exposure of 600 ppm will lead to a concentration of 12.4 μg/ml acetaldehyde in basal cells (step 1). The pH value will decrease by 0.49 units (step 2) which will cause degeneration of the olfactory epithelium (step 3) and will lead to replacement proliferation (step 4) of basal cells. Therefore, all steps critical for carcinogenesis are active at 600 ppm vinyl acetate. This leads to a clear increase of tumor incidence (Fig. 7). The model demonstrates that the mechanisms critical for carcinogenesis (steps 1–4) become active only when threshold concentrations of acetaldehyde and acetic acid are exceeded. These threshold concentrations will only be exceeded when vinyl acetate exposure occurs above certain levels (Fig. 7). In conclusion, two metabolites, acetaldehyde and acetic acid, are responsible for the toxic and carcinogenic effects of vinyl acetate. Both metabolites also occur endogenously. Only above certain threshold concentrations carcinogenicity can be expected. Therefore, risk assessment of vinyl acetate has to take into account quite different principles as for, for example, aflatoxin B_1 or heterocyclic amines where similar threshold mechanisms are not known.

Cross-References

- ▶ Toxicity Testing In Vitro: Regulatory Aspects
- ▶ Toxicological Risk Assessment

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Dose–Response Relationship and Extrapolation in Toxicology: Mechanistic and Statistical Considerations

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Werner K. Lutz, Roman W. Lutz, David W. Gaylor, and Rory B. Conolly

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W. K. Lutz (✉)
Institute of Pharmacology and Toxicology, University of Würzburg, Würzburg, Germany
e-mail: lutz@toxi.uni-wuerzburg.de

R. W. Lutz
Formerly: Seminar for Statistics, Swiss Federal Institute of Technology, Zürich, Switzerland

D. W. Gaylor
Formerly: National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR, USA

R. B. Conolly
Integrated Systems Toxicology Division, NHEERL/ORD, U.S. Environmental Protection Agency, Research Triangle Park, NC, USA

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Abstract

Controversy on toxicological dose–response relationships and extrapolation of an incidence to low dose can be the consequence of misleading data presentation, diverging mechanistic understanding, or lack of differentiation between a continuous response variable, such as any concentration of a biomarker, and an incidence derived from a binary response (yes or no?) in individuals (dichotomous variable). In this chapter, we address respective issues and illustrate them with examples for genotoxicity, mutagenicity, and cancer incidence. The rate of any interaction of a toxicant with a biological target molecule at low dose is proportional to its concentration. Linear extrapolation is therefore a reasonable default for rates of first-line interaction in the low-dose range. In toxicity testing however, (i) we do not measure rates of interactions but concentrations of biomarkers, and (ii) we deal with a dose range that usually expands to overt toxicity. Deviation from linearity is observed with increasing dose whenever saturation, inhibition, or induction of a process involved comes into play.

Keywords

Adenylyl cyclase · Central limit theorem · Carcinogenesis, chemical · DNA methylation · Genotoxicity · Lognormal distribution · Low-dose effect · Monte Carlo simulations · Repair · Stochastic modeling · Threshold · Tolerance distribution models

Introduction

A nonmonotonic shape of the dose–response curve may be observed as a special case of nonlinearity, if a background measure in untreated controls is decreased at low dose but increased at high dose. A dose response can appear as a threshold if two processes that affect the background level in opposite directions cancel each other out. A mathematical threshold, where there is no effect at all up to a defined breakpoint of the dose–response "curve," cannot be advocated for any continuous response measure. We use computational modeling to characterize how competing influences that are dominant over different dose ranges combine to generate different shapes. The situation is different for an incidence of a defined effect, e.g., a diagnosis of cancer. On an individual level, the response is given by a binary "yes or no." For dose response, each individual has its own "threshold dose" to switch from "no cancer" to "cancer"; the dose–incidence "curve" represents a staircase of individual threshold doses and reflects the tolerance

distribution in the examined population. Extrapolation to low dose therefore follows differences in individual susceptibility and cannot be predicted by the mode of interaction between toxicant and biological target. For complex endpoints of toxicity such as cancer, individual susceptibility is determined by numerous genetic and in-life factors, such as enzymatic activation and detoxification of endogenous and exogenous carcinogens, DNA repair, or cell cycle control. Multiplicative combination of the individual activity of these factors and application of the central limit theorem of statistics suggests that the tolerance distribution – and with this the dose–incidence relationship – is approximated by a cumulative normal curve against log (dose). Using this model for a dose–incidence extrapolation, the cancer risk drops faster than by linear extrapolation, the more we approach dose zero. In the last section, we combine a mechanistically supported nonmonotonic dose response with individual differences for the rate of the underlying counteracting processes. Monte Carlo simulations indicate that a nonmonotonic shape of a dose response for a biomarker, determined as an average of a dose group, does not exclude a monotonic shape for some individuals. An observation of a nonmonotonicity in animals cannot be carried over by default to a dose–incidence response in a human population.

Dose–Response Curve in Textbook

The usual representation of a dose–response relationship is the cumulative normal distribution against the logarithm of the dose (Fig. 1). It is based on the finding that a lognormal curve often provides a good fit to data of different types of response variables. This holds for continuous response variables such as any rate of a process

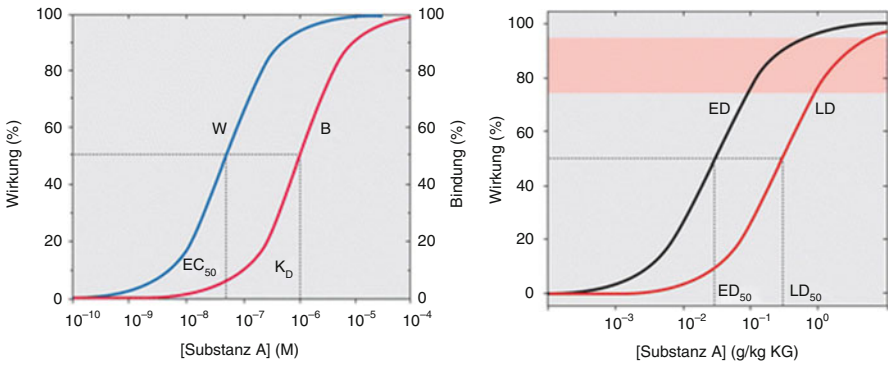

Fig. 1 Typical sigmoid shape of dose–response curves in textbooks of pharmacology and toxicology for different types of response variables, using a logarithmic scaling of the dose axis. *Left* (continuous response variables): ligand–receptor binding (*B*) and receptor-mediated response (*W*, "Wirkung") as a function of the logarithm of the ligand concentration (*substance A*). *Right* (dichotomous variable): incidence of a therapeutic effect (*E*) or of death (*L*) shown as a function of log (*dose*). (Reprinted with permission from Aktories/Förstermann/Hofmann/Starke, Pharmakologie und Toxikologie, 9. Auflage 2005, Abb. 1–4 and 1–7, Urban & Fischer, München)

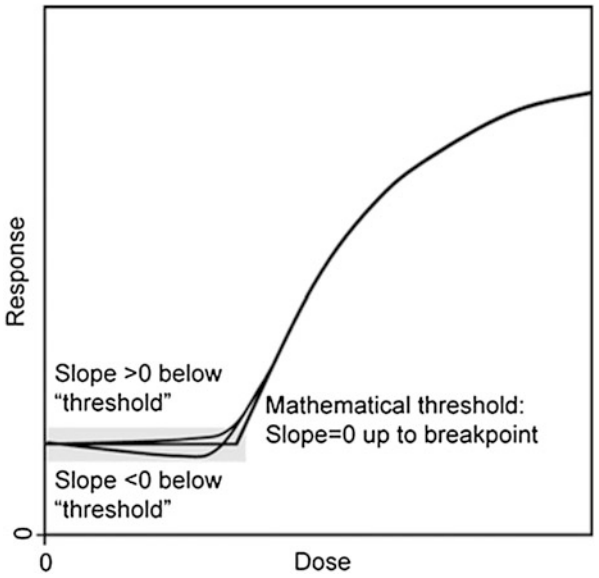

Fig. 2 Three shapes of dose–response curves that could be interpreted as indicating some type of "*threshold*": linear–sublinear (a nonzero slope at low dose, bending up at an undefined threshold), mathematical threshold (*slope* = 0 up to a defined breakpoint dose), and nonmonotonic dose response (*slope* < 0 *below* the "threshold"). (Reprinted with permission Lutz and Lutz 2009)

or concentration of a biomarker, as well as for an incidence of a defined effect, which is based on a binary (yes-or-no) response in individuals.

For an extrapolation to background (dose zero), the logarithmic dose scaling may be misleading because the sublinear appearance at the low-dose end may be interpreted as indicating a threshold. For an appropriate discussion of dose–response curves, it is crucial to understand (i) the consequences of logarithmic scaling of the dose axis and (ii) to clearly define the term "threshold."

Figure 2 shows that "threshold" could mean different things (Lutz and Lutz 2009). One curve starts with a positive but statistically insignificant slope and bends up at the "threshold dose"; the second is a mathematical threshold that is defined by an initial slope zero, followed by slope > 0 at some breakpoint; and the third has an initially negative slope, which results in a nonmonotonic shape over the whole dose range. We will later discuss mechanism and conditions that may lead to the different types of "threshold."

The Logarithm "Catch"

First of all, we must caution against the use of a logarithmic scale for the dose axis in connection with a discussion of dose–response extrapolation (Lutz et al. 2005). Problem #1: Since log (0) is indefinite ("- ∞"), the response measure of

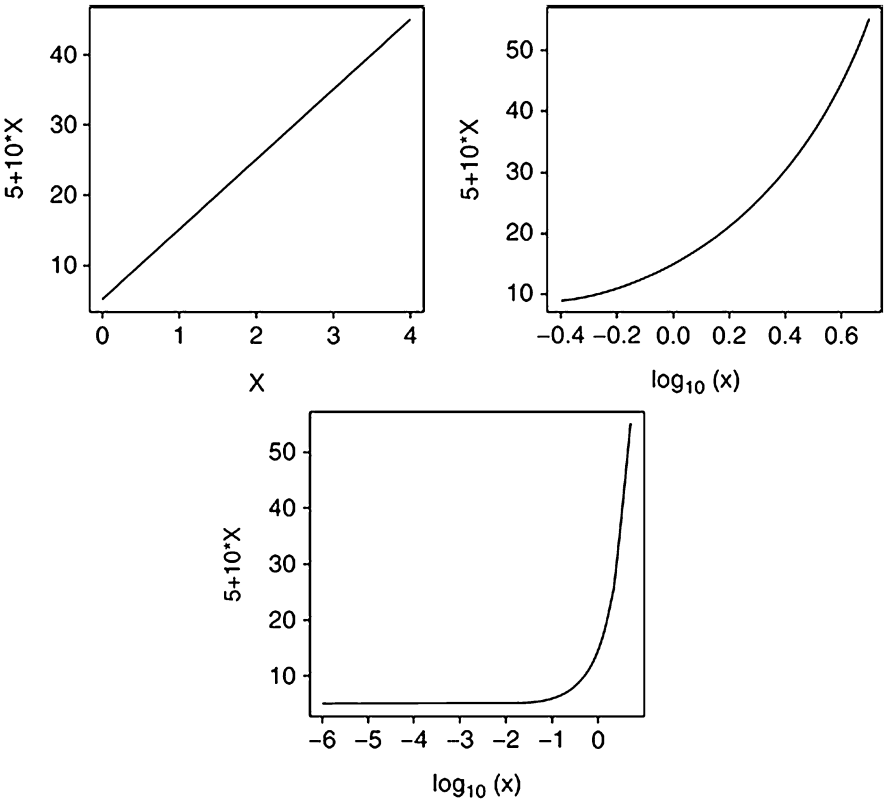

Fig. 3 Logarithmic representation of the dose axis distorts the straight line of a linear dose response into a threshold appearance. All three representations show the same linear dose–response relationship $y = 5 + 10{*}x$. *Left*: arithmetic dose scale; *center*: log scale spanning one factor of ten for dose; *right*: log scale spanning seven factors of ten down to dose 10^{-6}

the control group at dose zero cannot be plotted in the same graph together with the treated groups, and visual inspection of the dose range of extrapolation to background is not possible. Problem #2: Logarithmic scaling of the dose axis distorts a straight line ("linearity") into a threshold-like curve. Figure 3 shows different representations of the same linear function $y = 5 + 10 * x$. An arithmetic scale is used for the left panel. The center panel uses a logarithmic scale and spans doses between 0.4 and 4. A sublinear shape is seen. The right panel spans six orders of magnitude down to dose 10^{-6}. The dose groups that show an increase above control are compressed into one factor of ten and appear with a steep slope. Such a delusive appearance of a threshold still shows up in publications. It is easily generated by the use of doses that are many orders of magnitude below the no-observed-effect level.

The low-dose part of the sigmoid shape shown for receptor–ligand binding ($R + L \rightarrow RL$) in Fig. 1b is another example of the result of logarithmic dose scaling.

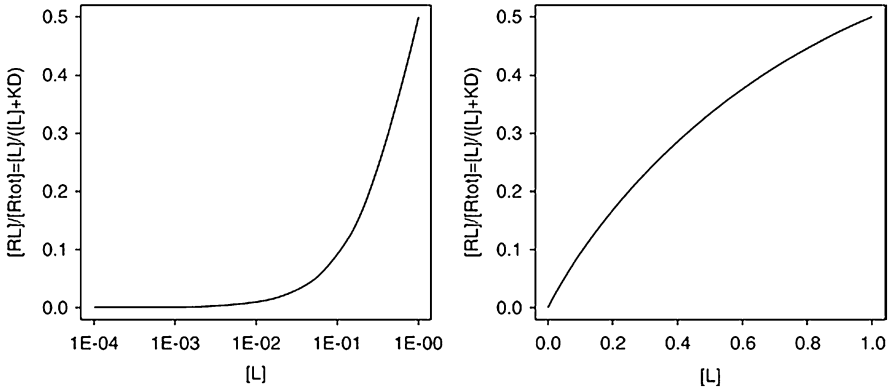

Fig. 4 Low-dose part of receptor–ligand (RL) complex formation as a function of ligand concentration $[RL]/[R_{tot}] = [L]/([L] + K_D)$ for the dissociation constant $K_D = 1$, shown on a logarithmic scale (*left*; as used in Fig. 1) and on an arithmetic scale (*right*)

The underlying function [RL]/[Rtot] = [L]/([L] + Kd) is not sigmoid but a hyperbola, and linearity is a good approximation at low concentration of ligand [L]. Figure 4 shows this function on a logarithmic and an arithmetic scale (left and right panel, respectively). Note that Michaelis–Menten kinetics of enzyme reactions follows the same function. This also means that the rate of enzymatic product formation is approximately proportional to the substrate concentration at concentrations below the Michaelis constant.

Conclusions

The fact that logarithmic scaling of the dose axis provides good data fit by a cumulative normal distribution, data both for continuous variables and for incidences (dichotomous variable), is misleading in two ways. On the one hand, the sublinear appearance of the low-dose part can mimic a threshold even for a linear dose response; on the other hand, it is suggestive of the misconception that the sigmoid shape of a dose–incidence relationship adheres to the same principles as a dose response for a continuous variable.

Continuous Response Variables

Linearity as Default Extrapolation for Rates of First-Line Interactions

Many biomarkers measured in toxicity testing are concentrations, e.g., products of physical or chemical interaction of a toxicant with a biological target (binding to a receptor or an enzyme; reaction with protein or DNA). According to the law of mass action, the rate of interaction is approximately proportional to the concentration of the reaction partners at low dose. Linear extrapolation is therefore an appropriate default for the low-dose end. This includes situations of complex metabolic

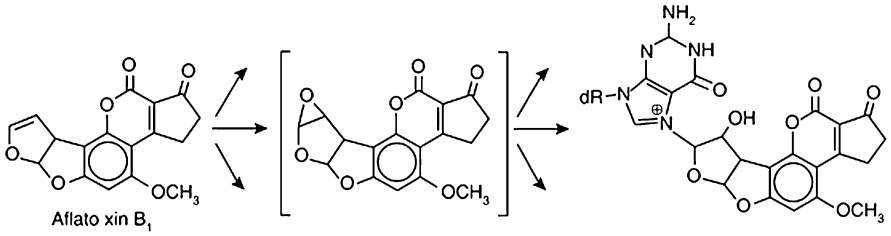

Fig. 5 Two-step formation of a guanyl-7-adduct in DNA by the carcinogenic mycotoxin aflatoxin B_1 via metabolic activation to the chemically reactive epoxide. *Up*-and- *down arrows* indicate competing processes of detoxification

activation where the toxic reaction product is the result of multiple steps and includes competing reactions. In Fig. 5, the mycotoxin aflatoxin B_1 (AFB_1), a potent hepatocarcinogen, is shown to react with the seven-position in guanine to form the respective promutagenic DNA adduct. Metabolic activation to the chemically reactive, electrophilic epoxide (in brackets) is a necessary intermediate step. The up-and-down arrows indicate that a number of concurrent reactions (other pathways of elimination; reaction with other nucleophiles, e.g., water or glutathione) take place. All reactions – toxification as well as detoxification – are approximately proportional to the concentration of the reactant as long as capacity-limited processes are not approaching saturation.

Considering extrapolation of a biomarker to low dose, it means that reducing the dose by a given factor is expected to result in a reduction of the biomarker by the same factor. For DNA-adduct levels, for instance, this also means that the rate of formation cannot drop to zero at any low dose. A mathematical threshold for a dose response as shown in Fig. 2 is therefore not possible. As an example of linearity down to the ng/kg dose range, DNA adducts in the liver of rats treated with [^{3}H]AFB_1 of high specific radioactivity decreased in a dose-proportional manner for all treatment scenarios at dose levels below 100 ng/kg per day (Fig. 6).

Deviation from Linearity Due to Saturation of Processes that Modulate Biomarker Levels

Toxicity studies usually include dose levels beyond the range of proportionality for the reaction rates that determine the response measure. Deviation from linearity is therefore the rule rather than the exception. For biomarkers of genotoxicity, one important mechanism that results in sublinear deviation of the dose response for mutation is saturation of DNA repair. Figure 7 shows the formation of a GC → AT base-pair substitution mutation resulting from methylation of guanine at the O^6-position (G∗). The full process of mutagenesis requires two rounds of DNA replication. It starts with mispairing of G∗ with thymine (T) at the first round, followed by

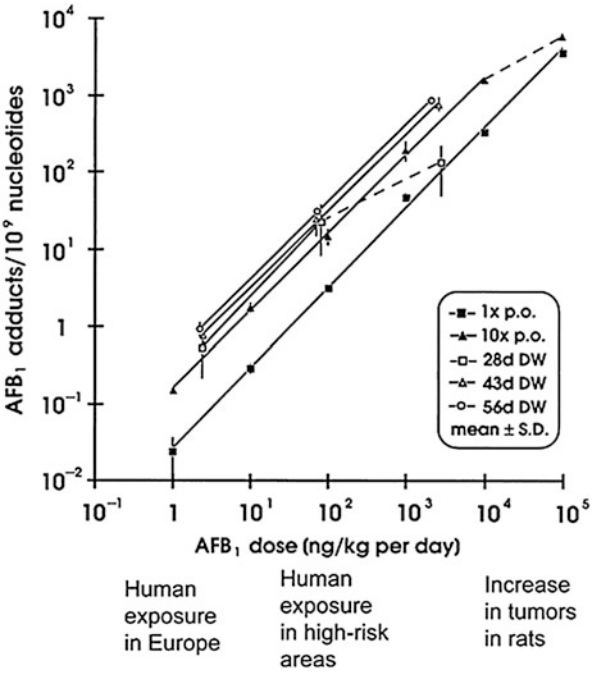

Fig. 6 Linear dose–response relationships at the low-dose end for [^{3}H]aflatoxin B_1-DNA adducts in rat liver. Single and multiple daily oral dosing, as well as application in drinking water (*DW*) for up to 56 days. Note the *double-log plot*: slope = 1 for all *full lines* are indicating proportionality between dose and response, i.e., linear dose response

the correct pairing of T with adenine (A) in the second round. Repair is possible at all stages of the process. At low dose, i.e., at slow rate of DNA-adduct formation, repair may be proportional to the damage, so that mutation rates stay low. The resulting slope of the dose-mutant frequency relationship is positive, but may not be significant. With further increase in dose, repair will become saturated, which results in a steep increase in slope for mutant formation.

Superposition of the rates of formation and repair of DNA are shown schematically in the left panel of Fig. 8. It shows a linear dose response for the rate of DNA-adduct formation (dashed line) and a saturation curve for DNA repair (dotted line). The difference between the two curves (adduct formation minus repair; full line) assumes a sublinear shape for the dose response for mutagenicity.

This situation is considered the mechanistic basis of a threshold-like dose response for the mutagenicity of ethyl methanesulfonate (EMS) in mice. The respective data had been collected in the follow-up of a drug contamination by this ethylating agent (Lutz 2009). Figure 9 shows the induction of lacZ mutants in MutaMouse® treated daily for 28 days with EMS. At low dose, DNA ethylation was probably repaired almost as rapidly as it had been formed. When the enzymatic

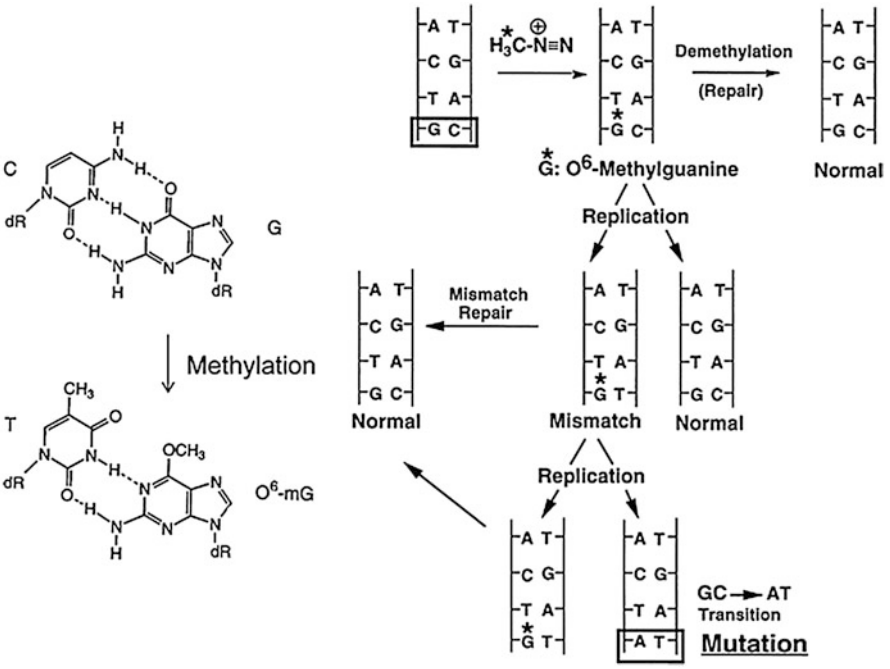

Fig. 7 *Left*: methylation of guanine (*G*) at the O^6-position and mispairing of O^6-mG (*G*∗) with thymine (*T*) instead of cytosine (*C*). *Right*: from a GC base pair to AT, i.e., a transition mutation requiring mispairing and two rounds of DNA replication

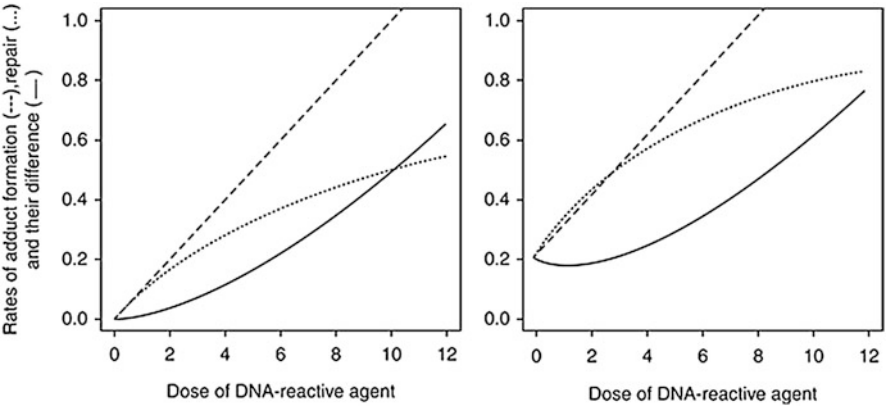

Fig. 8 *Left* panel: schematic representation of the superposition of a dose linear increase for adduct formation (*dashed line*) by a saturable rate of DNA repair (*dotted line*). The result is a sublinear curve for mutant frequency as a function of dose (*full line*). *Right* panel (includes background DNA damage at dose 0): The slope for exogenous adduct formation is the same as in the *left* panel. The repair activity is assumed to be induced (steeper slope at low dose) and active also on background adducts. Superposition results in a nonmonotonic dose response for mutant frequency

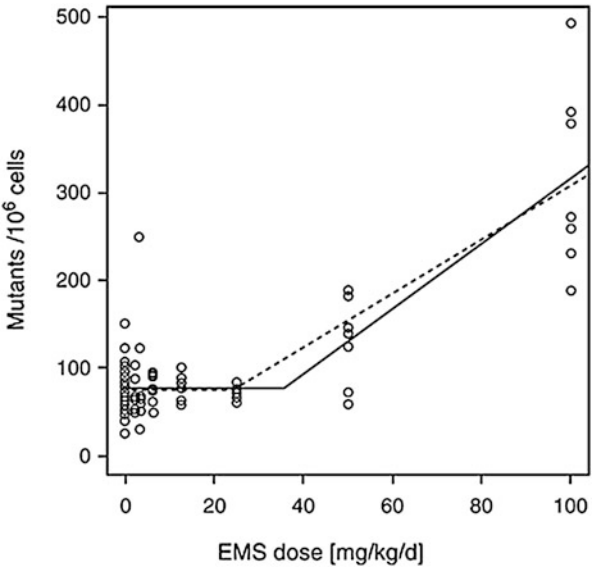

Fig. 9 Dose response for lacZ mutant frequency in bone marrow cells of MutatMouse™ treated daily for 28 days with ethyl methanesulfonate (EMS; CH_3–SO_2–O–CH_2–CH_3). *Circles* represent individual mice. The *full line* indicates the best fit by a hockey stick model; the *dashed line* represents the lower limit of a two-sided 90% confidence interval for the respective breakpoint (23 mg/kg per day). (Reprinted with permission Lutz and Lutz 2009)

DNA repair came into saturation with further increase in DNA ethylation, the dose–response curve for mutant induction bent upward. A hockey stick threshold model provided much better fit to the data than a linear dose response and showed a lower limit of a 90% confidence interval for a hypothetical breakpoint at 23 mg/kg per day (dashed line) (Lutz and Lutz 2009).

Nonmonotonic Shape if Background Is Reduced at Low Dose

In view of the general understanding that both adduct formation and repair are approximately proportional to low dose and the fact that repair always lags somewhat behind, one would expect minute, though positive slope for mutant induction also below the "threshold dose." In our example, however, linear regression of the mutant frequency data shown in Fig. 9 below the "threshold" shows a slightly negative slope (Fig. 10). It appears as if treatment of the mice with ethyl methanesulfonate below the putative threshold dose had resulted in a minute reduction of the background mutant frequency. If true, how could this be explained mechanistically?

DNA methylation of guanine by S-adenosyl methionine forms an important part of promutagenic background DNA damage. In view of the high mispairing

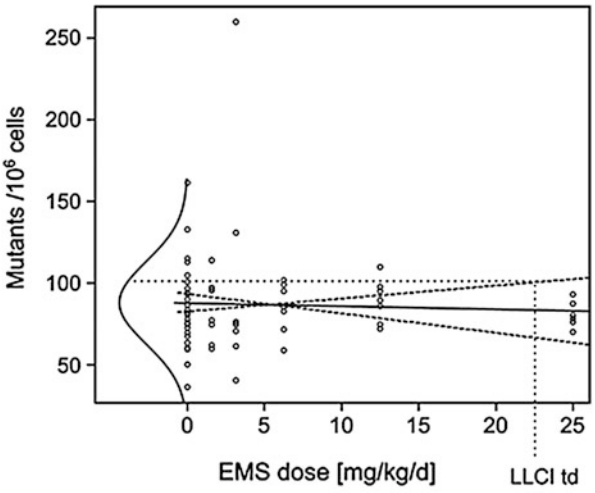

Fig. 10 Linear regression of the dose–response data below the threshold dose for lacZ mutant induction by ethyl methanesulfonate shown in Fig. 9 (see respective legend for experimental details). The best estimate of the slope is imperceptibly negative (*full line*). *Dashed lines* indicate the limits of a 90% confidence interval for the slope. The *horizontal dotted line* connects the upper bound of the regression at the threshold dose to the variance of response in control animals shown as a normal distribution. (Reprinted with permission Lutz and Lutz 2009)

potency of O^6-methylguanine, inducible repair has evolved to limit this dangerous type of DNA damage. The negative slope could therefore be explained by the hypothesis that O^6-ethylguanine, a DNA adduct similar in structure to O^6-methylguanine, induced DNA repair even at lowest doses of EMS. If the induced repair was not only active on DNA ethylation but also on background DNA methylation, one could explain the negative slope for mutant frequency. Over a wide dose range then, a nonmonotonic shape appears because of saturation of the induction of repair.

This hypothesis is illustrated schematically in the right panel of Fig. 8. It differs from the schema in the left panel in that it includes a background DNA damage and exhibits a steeper initial slope of repair due to additional induction of repair by DNA ethylation. Superposition of the linear dose response for the formation of adducts by the saturable rate of repair now results in a nonmonotonic shape for total DNA damage.

Confidence Limits on Low-Dose Effect and Comparison with Background Variation

While linear regression shows a negative, while not statistically significant, slope as best fit to the data, the true slope could also be positive or more negative. This is indicated in Fig. 10 by the dashed lines that show upper and lower limits of a

90% confidence interval for the slope of the linear regression. The question now is whether induced mutant frequency at the threshold dose of 23 mg/kg per day would be of concern if the upper limit of the confidence interval for the slope were true. This can be discussed on the basis of a comparison of the hypothetical increase in mutant frequency with the variation in background observed in the 27 control mice. The horizontal dotted line in Fig. 10 intersects at percentile 72 of a normal distribution fit to the data points, which allows the conclusion that even a statistically unlikely positive slope for mutant frequency would vanish within less than one standard deviation of the background variation.

Conclusions

The dose–response curve for continuous response variables of early biomarkers of toxicity is the result of a superposition of a number of processes that contribute to the response measure. Each single process shows a monotonic dose response that is approximately linear at low dose and usually saturates with increasing dose. Superposition of the contributing dose responses results in sublinear or supralinear deviation from linearity. If one of the processes reduces the background response level, a nonmonotonic shape may also be observed. A mathematical threshold, where slope zero changes at a defined breakpoint of the curve to slope > 0, can hardly be explained by a biologically based mechanism. For practical purposes, however, i.e., to provide an estimate and its confidence limits for the point of transition, a simple statistical threshold model such as the hockey stick model might be useful.

Mechanistic Background of Nonmonotonic Dose Response

Several scenarios that can give rise to nonmonotonic dose responses had been addressed before publication of the EMS data (Conolly and Lutz 2004):

1. Formation of cyclic AMP as a function of the binding of phenylisopropyladenosine to adenosine receptors: Data showing a nonmonotonic shape are explained by the antagonistic action of the adenosine receptors A1 and A2, given the differences in ligand affinity and efficacy of signal transduction. A1: antagonistic, high affinity, low efficacy; A2: agonistic, low affinity, high efficacy.
2. Androgen-mediated gene expression: Combined exposure to native androgen and a synthetic analog interacts competitively at the androgen receptor to form a series of homo- and heterodimers with differing abilities for promotion of gene expression.
3. DNA adducts and mutation: Induction of repair also repairs DNA damage due to a background process. This theoretical example in fact predicted the observations for ethyl methanesulfonate and lacZ mutant induction in transgenic mice discussed above.

Table 1 Examples of nonmonotonic dose response for processes that are dominated differently over different dose ranges. Response is below background at low dose and returns to background (for androgen-mediated gene expression) or is above background (other three examples) with increasing dose (Conolly and Lutz 2004)

Endpoint	Toxicant or ligand	Dominant influence at low dose	Dominant influence at high dose
Activity of adenylyl cyclase: Formation of cAMP	Phenylisopropyladenosine	Adenosine A1 receptor	Adenosine A2 receptor
Androgen-mediated gene expression	Hydroxyflutamide (in the presence of dihydrotestosterone)	Homodimers (dominant at low and high dose)	Heterodimers (dominant at mid-dose)
Total DNA damage (endogenous plus exogenous)	DNA adduct-forming agent	Induction of DNA repair	Exogenous DNA adducts
Mutation	DNA adduct-forming agent	Cell cycle checkpoint	Exogenous DNA adducts

4. Cell cycle checkpoints: DNA damage activates checkpoints in the cell cycle. Long-duration checkpoints provide additional time for DNA repair before DNA replication can fix the damage as a mutation.

The four examples, though diverse, are all characterized by the presence of more than one influence on the shape of the dose–response curve, with each influence being dominant over a different range of doses (Table 1).

It is possible within these four examples to distinguish two classes of mechanisms that give rise to nonmonotonic dose response. The latter two examples involve adaptive responses of the exposed tissue – induction of DNA repair and activation of cell cycle checkpoints. The first two examples – modulation of adenylyl cyclase activity and androgen-mediated gene expression – do not involve adaptation. Rather, they reflect constitutive biology. Adaptation requires some amount of time, hence any appearance of a nonmonotonic dose response has a temporal aspect – nonmonotonicity will not be seen if the interval between exposure and measurement of the relevant endpoint is too brief. For risk assessment, we are usually concerned with longer-term exposures, so that nonmonotonic responses on all kinds of adaptation must be considered relevant.

Computational modeling can be used to characterize how these influences combine to generate different dose responses, including nonmonotonicity (Conolly and Lutz 2004). This involves "parameter sweeps" where the value of the key parameter is varied across a range to produce a corresponding set of dose–response curves. As an example, for nonmonotonicity due to induction of DNA repair, a sweep on the parameter "induction of DNA repair" was conducted (Fig. 11). When the efficacy of induction is low (panel B; level 1), the dose–

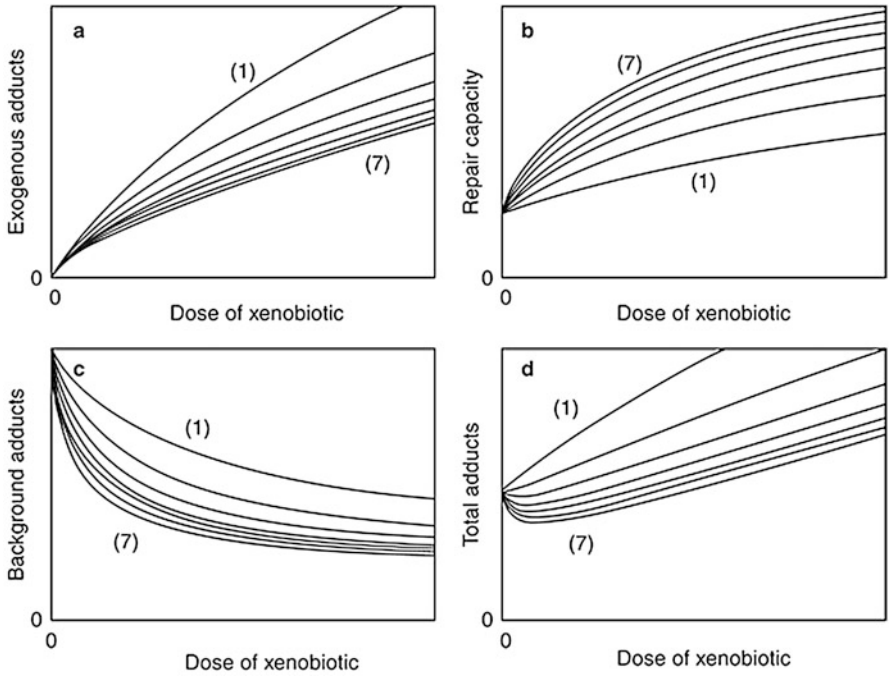

Fig. 11 Modeling dose–response relationships for DNA adducts as a function of dose of an exogenous mutagen (**a**), modulated by different levels (*1–7*) of repair induction (**b**). (**c**) Decrease of background DNA adducts due to the induced repair. (**d**) Total adducts (background plus exogenous) obtained by superimposition of (**a**) and (**c**). (Reprinted with permission Conolly and Lutz 2004)

response curve for total adducts is monotonic (panel D, showing the sum of exogenous and background adducts). High levels of induction (levels 4–7) generate nonmonotonic curves of increasing degree. Interestingly, an intermediate efficacy (level 3) leads to a dose–response curve where, at low dose, the increase in the adduct burden due to the xenobiotic is closely balanced by the induction of repair capacity, resulting in a threshold-like curve.

It is tempting to speculate that this result explains the data for ethyl methanesulfonate and lacZ mutation (see Figs. 9 and 10). The data are consistent with an intermediate efficacy for induction of a repair process that acts on both the background burden of promutagenic DNA damage and the damage due to ethyl methanesulfonate. Differentiation between (i) a monotonic curve with a shallow slope > 0, (ii) a seeming threshold, and (iii) a weakly nonmonotonic curve must be based on plausible mechanistic considerations. Data fitting by different models may find the statistically best fit, but this is no proof of the true shape of the dose response.

Similar results are obtained for the other three cases listed above (Conolly and Lutz 2004). Sweeping on a key parameter leads from a monotonic dose response, through an intermediate, threshold-like regime to a clearly nonmonotonic response.

These results suggest that the conditions, under which nonmonotonicity arises, may be only subtly different from those generating monotonic responses. It possibly involves no more than a quantitative difference in one of the background components of the effect under study.

"Incidence" as a Different Type of Response Variable

A Dose–Incidence Relationship Reflects Differences in Susceptibility

The risk of an exposure-related increase in a defined disease is measured as an incidence in a group of animals or humans. Each individual can either manifest this effect ("yes"; response value 1) or not show the effect ("no"; value 0). The incidence is given by the fraction or percent of responders in the group and increases with dose.

Figure 12 shows a hypothetical example of the dose–incidence relationship for the effect of alcohol on a group of ten humans. The yes-or-no criterion of toxicity is defined as the loss of balance to keep walking straight on. The group is given increasing volumes of wine at weekly intervals, and the test is made after 15 min. The graph shows that one individual manifested the adverse effect already when the dose increased from 100 to 150 mL. At the other end of the dose response, it took

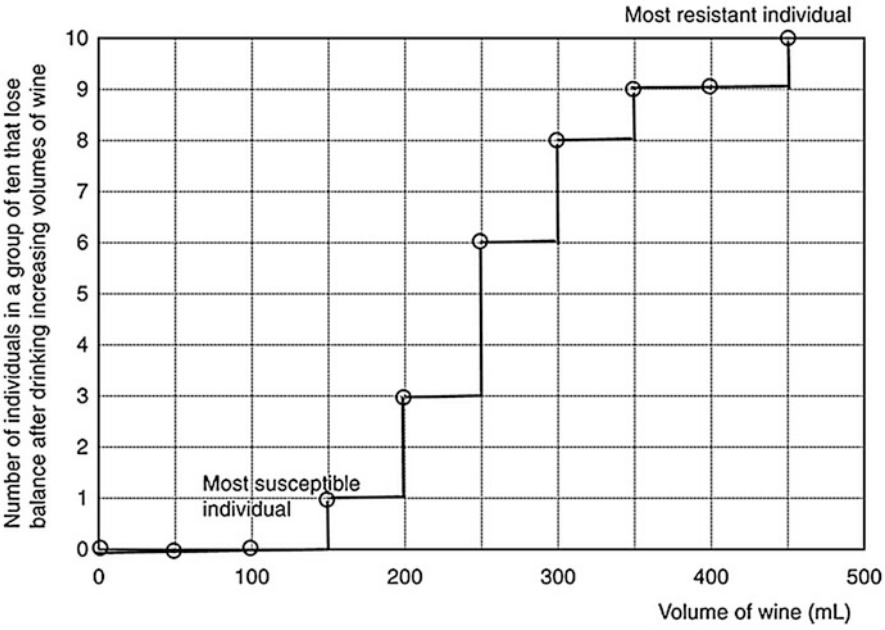

Fig. 12 Representation of a dose–incidence relationship for the effect of alcohol on a group of ten individuals. Dose steps: 50 ml of wine. Each individual has its own threshold dose range to lose balance

more than 400 mL to knock out the most tolerant individual. In other words, the threshold dose for the most susceptible individual was somewhere between 100 and 150 mL; the threshold dose for the most tolerant individual was between 400 and 450 mL. The resulting "curve" therefore represents the distribution of susceptibility (or tolerance) of the individuals in the group exposed.

The problem of risk extrapolation to low dose therefore boils down to the question about whether individuals in a large population show a lower threshold dose than observed in a small group of ten. In order to answer this question, we must investigate the criteria that are responsible for differences in susceptibility. For our example of tolerance of the acute effect of alcohol on the equilibrium, the most important criterion is the volume of distribution for ethanol. Since this is largely determined by the body weight, the most susceptible individual was probably a slim female, the most tolerant a heavy male. Other factors that you may mention are stomach content at the time of drinking (rate of absorption) and habituation to alcohol. Knowledge of the type of interaction of alcohol with its biological target(s), on the other hand, does not help predict the shape of the curve in the dose range of extrapolation. Information on the molecular mode of action is of interest only in the search of factors that may modulate the susceptibility. This limitation of the usefulness of mechanistic information for dose–incidence relationships is not commonly recognized.

Conclusions

As opposed to the situation of continuous response variables of biomarkers, a dose–incidence relationship is not a smooth curve but a flight of steps that represents the sequence of individual threshold doses to switch from "no" to "yes." The flight of stairs reflects the tolerance distribution in the respective group of individuals. Mode of action does not account for the shape of the dose–incidence relationship, but its knowledge may help define susceptibility factors, characterize and model their distribution in the population, and identify susceptible groups and individuals.

Chemical Carcinogenesis and Cancer Incidence

Tumor induction is a complex process with numerous modulatory factors that determine the individual's probability to manifest the disease after carcinogen exposure. Figure 13 shows a number of factors in rectangular boxes that express important interindividual differences: metabolic activation of a carcinogen, metabolic detoxification, rates of DNA repair and replication, inheritance of activated cancer genes or inactive tumor-suppressor genes, and immune surveillance, to name a few. How should these factors and activities be combined to result in a susceptibility expressed as an individual threshold dose? An example with two factors could illustrate the approach. Assume two individuals who differ by a factor of two for both the rates of detoxification of the aflatoxin epoxide and of repair of the respective DNA adduct. As a consequence, the rate of mutation will be four times as high in the individual with the lower activity for detoxification and DNA repair. In order to generate the same rate of mutation for both individuals, the aflatoxin dose has to be reduced by a factor of four for the more

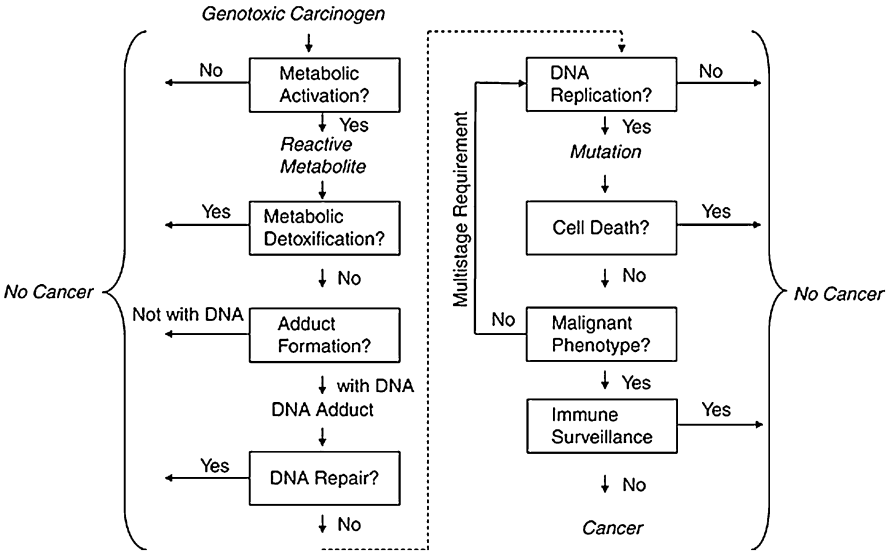

Fig. 13 Schematic representation of the process of chemical carcinogenesis by a genotoxic carcinogen. The *boxes* show factors for which individuals express different activity, which modulates the rates of the steps towards the manifestation of cancer

susceptible individual. As a conclusion, for quantitative combination of susceptibility factors, individual rates have to be multiplied.

Multiplicative Combination of Susceptibility Factors Results in a Lognormal Distribution

The central limit theorem of statistics states that *sums* of a large number of independent random variables are approximately normally distributed. The Galton board (1889) shown in the top left panel of Fig. 14 illustrates the principle, for the simplest situation of the sum of ten binary variables, where balls that can fall either to the left or to the right (Limpert et al. 2001). *Multiplicative* combination of the ten variables calls for different shapes of the triangles, as illustrated in the top right panel of Fig. 14; the distribution now has a positive (right) skew. The bottom panels demonstrate that logarithmic transformation of the x-axis reverts the right skew to the symmetry of the normal distribution defined by its mean and a *multiplicative* standard deviation.

To implement these finding for a discussion of a dose–cancer incidence relationship means that the x-axis represents the dose axis; balls represent human individuals with different threshold doses of carcinogen to get cancer. The binary factors chosen for the Galton board obviously do represent the world of biology. The factors that modulate the rate of chemical carcinogenesis as shown in Fig. 13 can assume different types of distribution and variance. It will be a future task to collect the respective information

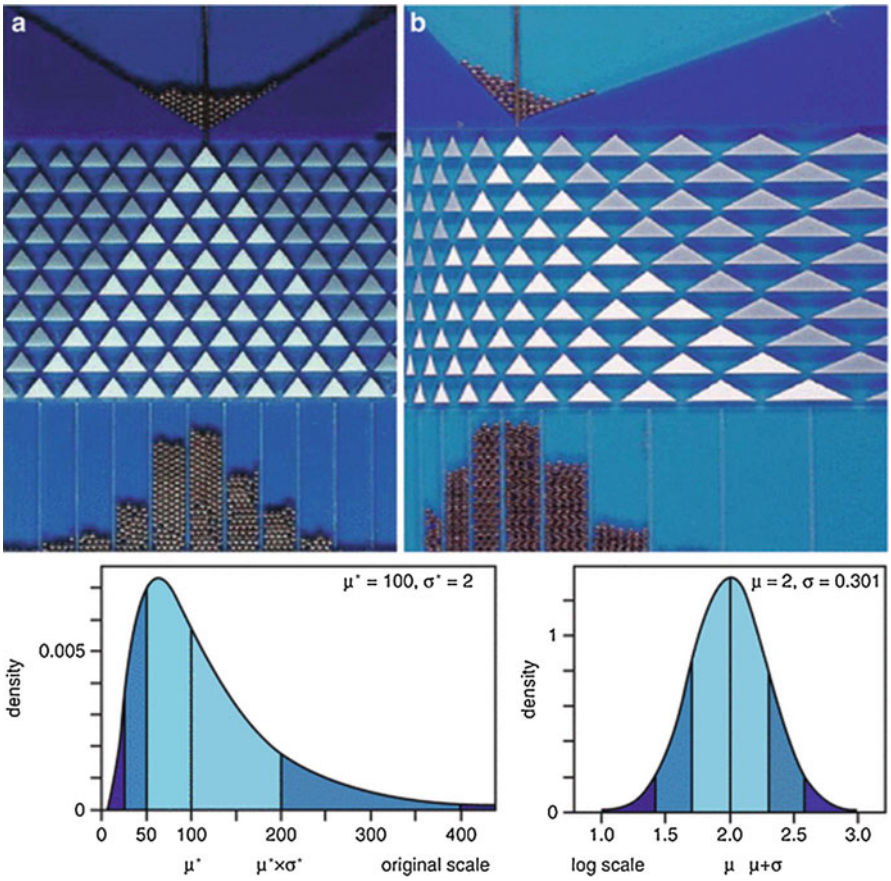

Fig. 14 *Top*: the Galton board (1889). Physical models illustrating the central limit theorem of statistics. *Left*: additive superposition of ten "good or bad" random susceptibility factors. *Right*: multiplicative superposition of factors, which generates a right-skewed distribution. *Bottom*: using a log scale, the *right* skew (*left panel*) converts back to the normal distribution (*right panel*). (Reprinted with permission Limpert et al. 2001)

in the human population. The larger the number of factors and the larger their variances, the larger will the multiplicative standard deviation of the lognormal distribution become.

Extrapolation of a Dose–Cancer Incidence Relationship

It Is Time for a Revival of the Lognormal Distribution for Cancer Risk Extrapolation

The first model employed by the US regulatory agencies to estimate a cancer risk at low dose utilized the Mantel–Bryan procedure (Mantel and Bryan 1961). Starting from animal data and considering a wider variability in the human

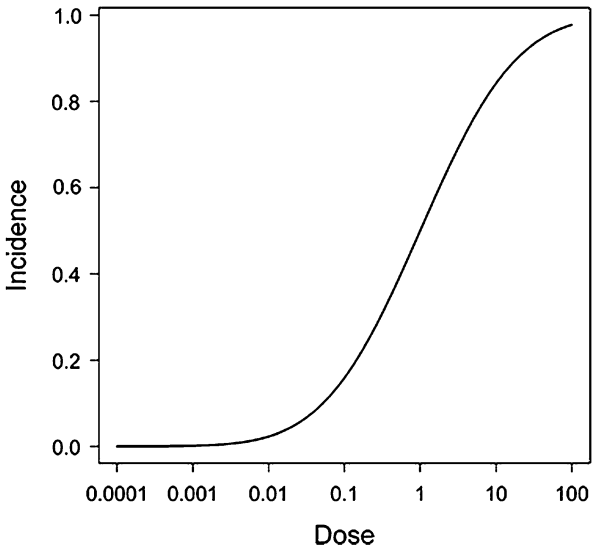

Fig. 15 Dose–cancer incidence relationship following on a lognormal susceptibility distribution. Carcinogenic potency $TD_{50} = 1$ (incidence 0.5 [50%] at dose 1). Multiplicative standard deviation $\log_{10}(10) = 1$, i.e., a dose -reduction factor of 10 results in an incidence reduction by 1 standard deviation (Mantel and Bryan 1961). The extrapolated cancer incidence for dose 0.1, 0.01, 0.001, and 0.0001 is 0.16, 0.023, 0.0014, and 0.00003, respectively (see also Table 2)

population, the susceptibility was suggested to follow a normal distribution against $\log_{10}$ (dose) with a conservative multiplicative standard deviation of 10 (called "slope 1" [$\log_{10}$ of 10]). In other words, reduction of the dose by a factor of ten was assumed to result in a decrease of the incidence by one standard deviation. The approach was abandoned because of the uncertainty associated with the assumption on the slope and because of arguments of the stochastic aspects of carcinogenesis.

Dose–response data for tumor incidence in humans are very limited. For lung cancer incidence as a function of cigarette smoking, the data available for British physicians were analyzed using different models, including a lognormal distribution. Best fit was achieved with a multiplicative standard deviation of 5.75 (Whittemore and Altshuler 1976). The drop in risk for drop in dose for this particular example is therefore steeper than when using the default assumption of Mantel and Bryan.

The dose–cancer incidence curve that follows a lognormal susceptibility distribution is shown in Fig. 15. As starting point it assumes a dose of 1 for a 50% tumor incidence; the drop in risk with drop in dose follows the conservative assumption of Mantel and Bryan. The graph shows the respective drop in risk from 0.5 to 0.16 (16%) with the first step in dose reduction by a factor of 10 and a reduction from 16% to 2.3% by another factor of ten. The graph does not allow visual assessment of the cancer risk with further decrease in dose. Table 2 provides this information down to 10^{-5} times the TD_{50}, both for the Mantel–Bryan

Table 2 Low-dose extrapolation of cancer incidence in a population of 100,000. Comparison between a linear and two lognormal dose–response relationships with different standard deviations. Assumptions: tumorigenic potency: $TD_{50} = 1$ dose unit; dose-reduction factor: 10

	Linear extrapolation		Lognormal extrapolation multiplicative SD = 10 (log_{10} probit slope 1)		Lognormal extrapolation multiplicative SD = 5.75 (log_{10} probit slope 0.76)	
Dose	Incidence in 100,000	Risk reduction factor	Incidence in 100,000	Risk reduction factor	Incidence in 100,000	Risk reduction factor
1 (TD_{50})	50,000		50,000		50,000	
0.1	5,000	10	15,865	3	9,412	5.3
0.01	500	10	2,275	7	425	22
0.001	50	10	135	17	4	107
0.0001	5	10	3	45	0.007	558
0.00001	0.5	10	0.03	110	0.000002	2987

assumption and the human lung tumor data for smokers. Comparison with a linear extrapolation allows the following conclusions: At relatively high-dose levels in the range of 0.1 times the TD_{50}, linear extrapolation drops faster than the lognormal extrapolations. The ranking reverses with every further dose step towards zero. At dose 0.0001 times the TD_{50}, for instance, linear extrapolation predicts a much higher incidence than following the lognormal curve postulated for lung cancer in smokers.

Tolerance Distribution Versus Stochastic Modeling

Knowledge on susceptibility factors for the rate of carcinogenesis as shown in Fig. 13 has increased dramatically in the last few decades. Major advances can be noted for the inheritance of mutant "cancer genes" of individual differences for DNA repair and metabolic activation and detoxification. The use of tolerance distribution models for cancer risk extrapolation therefore deserves a revival. Yet, a number of aspects of the process of chemical carcinogenesis may keep a stochastic element. For instance, the question of whether a DNA adduct is formed in a critical gene (oncogene or tumor-suppressor gene) or in an innocuous gene will not be fully predictable on an individual level.

Conclusions

A dose–cancer incidence relationship for a given population is predictable to the extent of our knowledge of the distribution of individual risk factors. Confidence limits will have to be widened to account for remaining stochastic aspects. These limitations do not invalidate the general statement that linear extrapolation of a treatment-related excess cancer risk to background incidence is inappropriate.

How to Incorporate a Nonlinearity of an Experimental Biomarker in a Dose–Incidence Relationship?

The threshold-type dose response shown above for mutant induction in mice treated with ethyl methanesulfonate (Figs. 9 and 10) leads us to the question how this knowledge can be used for a dose–incidence curve for humans exposed to this genotoxic agent. Since a dose–incidence curve is not directly dependent on mode of action but follows the distribution of tolerance within the population, the question must be addressed whether the factors that result in the deviation from linearity in the transgenic mice also operate in the human population. How is the activity of the protective factor(s) distributed among individuals? In the particular case of repair of DNA ethylation, it will be important to investigate whether there are individuals that may not benefit from this type of DNA repair and its induction.

Since DNA methylation is one of the most critical types of background DNA damage, an individual with little or no respective repair would probably accumulate lethal mutations already during fetal development. It could therefore be assumed that all newborn are able to repair DNA methylation to an extent required for survival and show a nonlinear dose response for exogenous DNA ethylation. Whether there is even a nonmonotonic shape as shown as an average response in mice will depend on the distribution in the human population of the inducibility of repair activity.

Monte Carlo Simulations to Differentiate the Dose Response for a Population Average Versus Individuals

Our model for induction of DNA repair is based on a normal distribution for the parameter that determines the efficacy with which DNA damage due to the xenobiotic induces DNA repair. Monte Carlo sampling allows prediction of dose–response curves for individual members of a population (Fig. 16). The four panels show how individuals in a population can differ in their response to genotoxicant exposure, given interindividual variation in ability to induce DNA repair. All types of dose–response curves are seen: monotonic, seeming thresholds, and nonmonotonic. Note that the mean and the lower confidence limit are monotonic, while the 95% upper confidence limit on the mean response is nonmonotonic. Similar results are obtained for the Monte Carlo version of the model predicting how activation of cell cycle checkpoints affects the rate of mutation (Conolly et al. 2005).

While computational studies and simulations are theoretical and while we know of no dataset that actually shows different behaviors of dose response in human individuals, we do think that the results suggest mechanisms by which individuals within a population may have quite different susceptibilities to xenobiotic stressors.

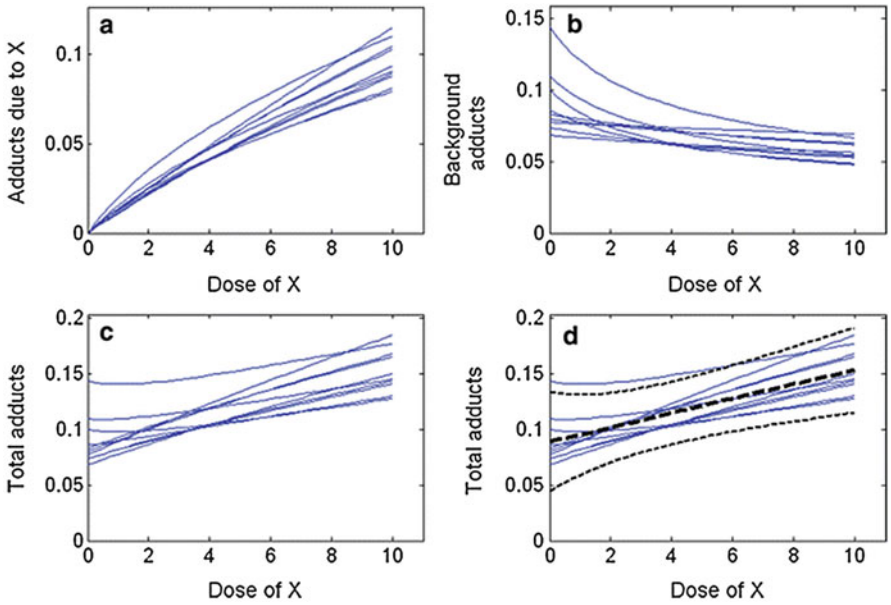

Fig. 16 Monte Carlo simulation with ten runs to generate dose–response curves for ten individuals who vary in their ability to induce DNA repair capacity. (**a**) Formation of DNA adduct from xenobiotic as a function of dose. (**b**) Background (endogenous) DNA adducts as a function of the dose of X. (**c**) Total DNA adducts (endogenous plus exogenous). (**d**) Same as (**c**) plus *dashed lines* to show the *upper* and *lower* 95% confidence limits and the mean for the population. (Reprinted with permission Conolly et al. 2005)

Conclusions

A nonmonotonic shape of a dose response shown for a population average does not exclude a monotonic shape for subpopulations or individuals. This limitation holds both for a dose response of a continuous biomarker and for a dose–incidence relationship. Nonmonotonicity cannot be considered a default for a population unless there are convincing arguments that all individuals meet the same quantitative criteria for the underlying modulatory factors.

Cross-References

► Benchmark Dose Approach in Regulatory Toxicology
► Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds
► Statistical Evaluation Methods in Toxicology

EPA Disclaimer This chapter has been reviewed by the United States Environmental Protection Agency and approved for publication but it may not reflect the views of the Agency and no official endorsement should be inferred.

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Importance of Exposure Level for Toxicological Risk Assessment

55

Hans Drexler and Anuradha Shukla

Contents

Abstract

A health risk for an individual due to an exposure to a hazardous substance depends upon the properties of the substance, the amount of the substance, and the susceptibility of the individual. If an individual is susceptible to a particular hazardous substance only the amount of the substance determines the risk resulting from the exposure to this substance. In the case of local effects the concentration of the hazardous substance in the environment and the duration of the contact determine the risk, with the exception of allergic reactions where the susceptibility is more significant. In the case of systemic effects only the internal exposure or dose is relevant for the risk. Therefore, it is important for the marker of exposure to be a good surrogate for the dose in the target organ. If appropriate methods are available a biological monitoring is more significant for risk assessment as compared to ambient monitoring. If biomarkers of effect are available not only the general but also the individual risk can be assessed.

H. Drexler (✉) · A. Shukla
Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine, University of Erlangen-Nürnberg, FAU, Erlangen, Germany
e-mail: hans.drexler@fau.de; hans.drexler@ipasum.uni-erlangen.de

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Keywords

Individual exposure · Susceptibility · Dermal absorption · Sensitization · Internal exposure · Effect monitoring · Protein adducts · DNA adducts

Introduction

Risk assessment is the quantification of the likelihood that a quantitatively defined exposure of an individual (or a group of individuals) to a given chemical might result in some adverse health effects. The probability depends on three elements: the risk factor itself (hazard or hazardous substance), the level and duration of exposure, and the individual susceptibility.

This can be described with the simple equation:

$$\text{Risk} = \text{Hazard} \times \text{Exposure} \times \text{Susceptibility}$$

The equation states that for an existing level of risk to be present, each of the three components must be different from zero (Manno et al. 2010). A risk assessment needs information about the hazard and the susceptibility and must be based on a valid exposure assessment.

Exposure assessment requires a monitoring of the concentration of the hazardous substances in the air or in materials, or the concentration of the substances or their metabolites in the body fluids of exposed persons. For this purpose it is necessary to use analytical methods which have been tested for reliability and practicability. An appropriate internal quality assessment as well as an external quality assessment of the applied methods is essential to assure the accuracy and the comparability of results. For example, the international program of the German External Quality Assessment Scheme (G-EQUAS) provides proficiency testing for most of the human biomonitoring parameters, which are commonly used for the assessment of the human exposure to chemicals (Göen et al. 2012).

If scientifically based threshold limits in water, food, or air are available the exposure assessment is often used in terms of a risk assessment for human health. This means that in cases where the threshold limits are exceeded it would result in a concrete risk to the individual. This is best possible for local effects caused by the hazardous substance. For a systemic effect, however, this would be justified only when the measured value in water, food, or air is a good surrogate for the effective dose in the human body.

Hazardous substances from the environment come into contact with the human body via mucous membranes, skin, lungs, and the gastrointestinal tract. The critical toxicity of a hazardous substance can be its local toxicity or its systemic toxicity (Fig. 1).

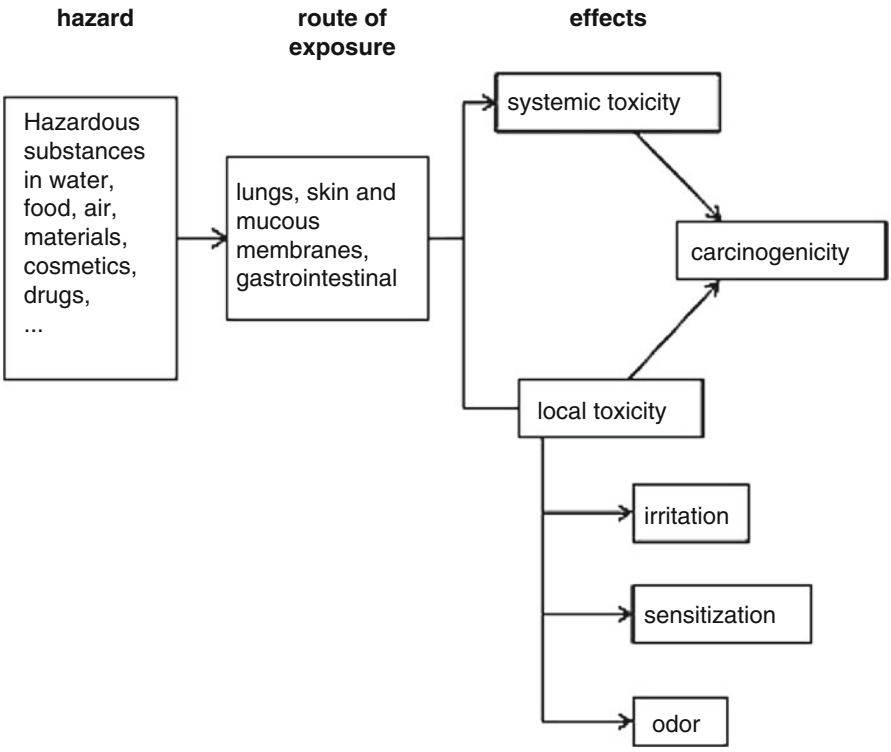

Fig. 1 Hazards and effects

Local Toxicity

Irritation

Exposure of persons to hazardous substances can cause irritation or erosion of the skin or the mucous membranes. These effects depend on the characteristics of the hazards and their concentration in the environment. At workplaces the concentration of a hazardous substance in air is of importance. Many occupational exposure limits for hazardous substances in air are based on irritative effects seen in man or in animals. For these hazards a time-weighted average threshold limit value (8 hours) would not be protective. Therefore, either short exposure threshold limit value for a 15-minute period or, in the case of a substance with a very high irritative potential, even ceiling threshold limits are evaluated. A guidance on how local irritancy data should be incorporated into risk assessment procedures, particularly with respect to the derivation of occupational exposure limits (OELs) has been published by Brüning et al. 2014.

Sensitization

Allergies caused by chemical substances affect mostly the skin (contact eczema, contact urticaria), the respiratory passages (rhinitis, asthma, alveolitis), and the conjunctiva (blepharoconjunctivitis). The kind of allergy is mainly determined by the chemical properties of the substance.

The development of a contact allergy of the delayed type is determined by several factors like the sensitization potential resulting from the chemical properties of the substance, the exposure concentration, the size of the exposed skin area, the duration and manner of exposure, the genetic disposition of the person, and, last but not the least, the state of the tissue with which the substance makes contact (DFG 2020). Therefore, apart from the concentration, susceptibility is also significant for skin sensitization. The size of the skin area correlates with the number of dendritic cells in the skin which come into contact with the allergen and, thus, also influences the risk of sensitization. A quantitative dermal exposure assessment that is valid is very difficult to do (Ness 1994) and not practicable for a routine exposure assessment. As the sensitization depends on the concentration of the substance there are ceiling concentrations for many allergens (e.g., formaldehyde, Nickel, fragrance) in consumer products. This should prevent sensitization; however, for already sensitized individuals the risk of an allergic reaction remains.

The allergic reactions of the airways and conjunctiva which take the form of bronchial asthma or rhinoconjunctivitis mostly involve reaction of the allergen with specific IgE antibodies and belong to the manifestations of the immediate type. Most respiratory allergens are macromolecules, mainly peptides or proteins. But low molecular weight substances can also produce specific immunological reactions in the airways. Allergic reactions of the immediate type can also cause systemic reactions and even anaphylactic shock. The development of allergies of the respiratory passages, like that of contact allergies, is dependent on a number of factors. In addition to the substance-specific potential for causing sensitization, the exposure period and the genetically determined disposition of the exposed person play a decisive role. Particular attention should be drawn to atopic diathesis which is characterized by an increased susceptibility to atopic eczema, allergic rhinitis, and allergic bronchial asthma with increased IgE synthesis (Schnuch et al. 2002). But the concentration of the allergen in air is very important for sensitization (Drexler et al. 2000) as well as for the provocations of symptoms (Drexler et al. 1999). So far it has been possible to evaluate health based threshold limit values only for a few allergens (isocyanates, flower).

For individuals who are already sensitized the individual susceptibility is very significant for the risk assessment. At least for the high molecular type-1 allergens persons with the so-called atopic diathesis have a considerably higher risk for sensitization than non-atopic individuals. After sensitization the hazards cause the allergic symptoms only in sensitized individuals. For non-sensitized individuals the susceptibility is zero, and according to the equation mentioned in the beginning of the text, the risk, therefore, is zero independent of the exposure.

Local Carcinogenicity

Carcinogenic substances can cause the risk of a systemic or a local cancer (e.g., asbestos, cadmium for lung cancer). In the case of a local cancer risk only the concentration in air is relevant for the extent of the hazard. It is, however, very important to differentiate between the total dust and the inhalable dust fraction which can enter the alveoli. Only the inhalable fraction of the dust is the fraction which is relevant to health. The aerodynamic diameter determines the fraction which enters the thorax (thoracic fraction). Some of the smaller solid particles and droplets are deposited in the tracheobronchial region or in the alveolar region. For this reason it is not enough to measure only the mass (mg/m^3 or ppm). The number of particles and their geometry are also very important for the resulting health risk (DFG 2020).

Systemic Toxicity

For hazardous substances which cause a systemic toxicity the quantification of biomarkers of exposure is a better surrogate than the quantification in food, water, materials, or air because only the amount of the hazardous substance which is incorporated into the body is relevant for the dose. The amount of the hazardous substance incorporated is very difficult to assess based only on the values in food, water, and other materials because the question as to how much of the hazardous substance is released and how much is absorbed can never be answered correctly. This is even true for hazardous substances in air because the amount of ventilation, the distribution of the hazardous substance during the time period, and the local distribution have a relevant influence on the dose. Also at work places it is often the additional skin contact which can be quantified only by means of a biological monitoring that is relevant.

In Fig. 2 the course of an externally caused health effect, for example, a lead induced anemia is shown. A part of the hazardous substance is absorbed from the environment and can be quantified as the internal exposure, like lead levels in blood in the above example. The amount which gets into the target organ correlates in most cases with the internal dose like lead in blood and lead in bone marrow. Early biochemical effects like the inhibition of the delta aminolevulinic acid (ALA) can be compensated without a biological effect. Chronic or intensive exposures result in initial biological effects (rise of ALA in urine) before the adverse health effect (anemia) is seen.

Biomarkers of exposure are the concentration of either a substance or its metabolites. There are various methods for analysis of hazardous substances in biological materials that have been published (DFG 2010). Important for the correct interpretation of the results is among others the knowledge of the half-life of the parameter which could be in the range of a few minutes (e.g., some solvents in blood) to many years (e.g., PCB, dioxins).

Detectable effect parameters like protein adducts and DNA adducts are also biomarkers of exposure as long as they have no role in the pathogenesis. The most

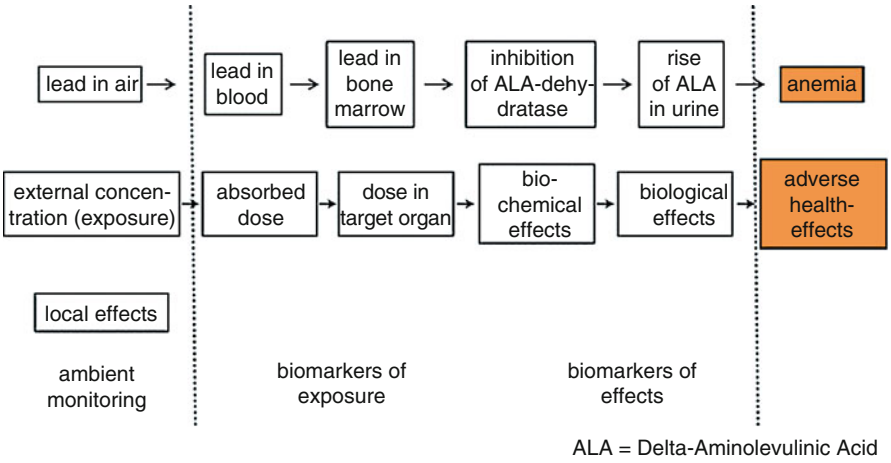

Fig. 2 Monitoring hazards and effects

commonly used protein adducts are hemoglobin adducts. The number of adducts with the amino acids in the hemoglobin is so low that the adducts do not influence the function of the hemoglobin. The advantage of the use of hemoglobin adducts is their half-life. Taking into account the life span of erythrocytes the hemoglobin adducts are used to assess the exposure during the last 3 months before the blood sampling. DNA adducts are biological target dose markers which reflect the exposure of the last 10 days before the sampling (Henderson et al., 1989).

Another advantage of the adduct biomarker is that one can estimate the proportion of the toxic metabolites. As a rule, it is the metabolite that is produced in phase 1 metabolism and not the hazardous substance itself that is responsible for the carcinogenic effect and for the formation of the hemoglobin adducts. Persons with a high activation (phase 1 metabolism) and a low deactivation (phase 2 metabolism) rate are more susceptible resulting in a higher cancer risk. For example, aromatic amines are activated by hydroxylation and deactivated by acetylation. The hydroxylated metabolite is excreted with the urine and forms the carcinogenic agent in the bladder. Under the same exposure conditions persons with a higher rate of acetylation have lower hemoglobin adducts and a lower risk of developing bladder cancer as compared to those with a low rate of acetylation.

Biomarkers of exposure quantify the dose whereas biomarkers of effect indicate early biochemical or functional alterations including a wide array of biological responses, ranging from physiological adaptation to disease. They represent a heterogeneous group of indicators and have different applications depending on the toxicological significance (Manno et al. 2010). The quantification of CO-Hemoglobin as biomarker of an exposure to carbon monoxide and the acetylcholine esterase activity as a biomarker of an exposure to inhibitors of this enzyme are well-known examples. Other endpoints, such as proteins in urine of subjects exposed to nephrotoxic solvents or metals, have been largely used as early indicators

of biological effect. This application requires, of course, that the target organ and preferably also the mechanism of chemical toxicity be known. Effect biomarkers used as early predictors of clinical disease can improve health risk assessment and contribute to implement new effective disease prevention in occupational and environmental settings, but they must be first validated. Validation also involves the clarification of the biomarker's toxicological significance, which means its relation with the chemical's mechanism of action and its ability to detect or predict a specific toxic effect (Manno et al. 2010).

Biomonitoring is used successfully in many environmental exposure studies either for the assessment of the current exposure of hazardous substances (Schwedler et al. 2017) or of the discovering of time trends of the exposure (Göen et al. 2018). In occupational studies, in addition to recording exposure, clinical parameters are usually also examined to determine health effects and the No Observed Adverse Effect Level (NOAEL).

Biomonitoring has also become an important instrument in routine diagnostics for physicians. In the field of occupational medicine, biomonitoring is the most important tool to assess the individual's exposure to specific chemicals, to characterize exposure pathways, and to assess potential individual risk factors. It is increasingly recognized that compliance with a limit value in the air for certain hazardous substances cannot exclude an unacceptably high exposure of workers (e.g., for lead), so that only the determination in biological material is the basis for health protection.

Cross-References

- ▶ Assessment of Background Exposure and Additional Exposure by Human Biomonitoring
- ▶ Exposure Scenarios in Toxicology
- ▶ Exposure Analysis for Indoor Contaminants
- ▶ Human Biomonitoring: Its Importance in Toxicological Regulation
- ▶ Uncertainty Analysis in Exposure Assessment-Relevance for Toxicological Risk Assessment

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Risk Characterization in Regulatory Toxicology

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Nikolaos Georgiadis and Maged Younes

Contents

Abstract

Risk characterization is the final step in the risk assessment process. All scientific data are summarized, reviewed, and evaluated in an integrated manner. Risk characterization should provide a clear description of the potential risk and outline the strengths and weaknesses of the whole risk assessment process. This includes a description of all assumptions and uncertainties of applied procedures, as well as a delineation of how the decision-making process.

N. Georgiadis
Toxicologist, European Chemicals Agency (ECHA), Helsinki, Finland
e-mail: nikolaos.georgiadis@echa.europa.eu; nikolaos.georgiadis@gmx.com

M. Younes (✉)
Global Publich Health, Mies, Switzerland

Formerly: Food Safety and Zoonoses, formerly: World Health Organization (WHO), Mies, Genève, Switzerland

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Keywords

Adverse-outcome-pathways · AOP · Characterization · Hazard · Risk · Risk-management questions · Uncertainty factors · Weight-of-evidence

Introduction

The goal of risk characterization is to provide decision makers with all the information necessary to take risk management actions in a logical and clear manner. The main question to be answered here is "Which effects, in the sense of a possible occurrence of a harmful effect, or in the sense of an increased risk, respectively, are linked with a certain given or expected exposure?" Typical risk management questions that require answers in the process of risk characterization are outlined in the text box below. The risk manager should have an understanding of, and a feel for, how exact risk predictions are, as well as to where the data described lie in the continuum from actual human data to data extrapolated from animal studies or in vitro experiments.

Some Risk Management Questions to Risk Characterization

1. What is the bottom line/final conclusion of the risk assessment?
2. Does the risk assessment provide sufficient information to justify regulatory action?
3. What is the range of uncertainty that characterizes the calculated exposure and the extrapolated number of potentially exposed individuals? Do we know if the calculated exposure corresponds to the actual one? Does the actual (or expected) exposure constitute a health (or environment) problem?
4. Which lacking data could give rise to criticism of the risk values or the risk management options?
5. Are there ongoing studies that could, eventually, provide lacking critical data in reasonable time?
6. Did the risk assessment undergo a peer-review process? If yes, by whom? What was the outcome?
7. Is there a possibility of "zero risk"? Was this really excluded?
8. Which key parameters drove the outcome of the risk assessment?
9. If studies were excluded from the risk assessment, which consequences did this have on its outcome? Why were these studies not considered?

It is also very important to identify vulnerable population groups or subgroups that are at particular risk under certain circumstances. An increased vulnerability could be the consequence of a higher exposure or an increased susceptibility. An example of an elevated exposure is that of population groups that consume very high amounts of fatty fish. Such groups may ingest higher amounts of, for instance, dioxins, polychlorinated biphenyls, methyl mercury, and other persistent compounds that accumulate in fat tissue. Children, in particular small children, constitute a group

with higher vulnerability towards a number of risk factors, since their organ systems and their physiological defense mechanism against toxic compounds are still not fully developed.

Elements of Risk Characterization

Hazard Characterization

Hazard characterization, the description of the potential to harm, requires an interpretation of all data on the toxicity and the dose–response relationship (Dybing et al. 2002).

While hazard identification is the process of determining whether exposure to a toxicant can cause an adverse health effect by assessing and integrating all the available information that may be in vivo studies, in vitro studies, in silico (QSAR, read across, etc.), epidemiological studies, and control clinical studies on humans, the objective of hazard characterization is to document the relationship between dose and response.

In the case of epidemiological studies, for example, it must be clarified if the exposed and control groups have been appropriately selected, if the length of the observation was adequate, if latent effects and confounding factors have been fully considered, if a causal relation between exposure and effect seems logical, and if the level of exposure/dose was adequately captured.

With experimental animal data, the main issue is about the integrity of the studies conducted. Here, a number of factors play a crucial role, among others if the studies have been conducted according to GLP principles (and if not, if an adequate and reproducible operative approach has been applied and described), how the choice of the test species and strain was made, the number of animals per dose group, the choice of dose or exposure levels, and the intervals between repetitive exposures, as well as the duration of the experiment. Often there are no data on certain endpoints. In such cases, it is important to evaluate, based on the existing information, to what extent the missing studies might change the outcome of the risk assessment.

At the onset, information concerning the completeness and the quality of the database is assessed. Studies are evaluated with respect whether or not they have been conducted according to accepted scientific and ethical principles and if they have been adequately assessed and documented.

An important step in hazard characterization is the identification of the critical effect (or critical effects). In some instances, several toxic endpoints are observed. The decision as to which of these effects can be considered as critical (and there may be more than one) depends on the severity of the respective toxicological endpoint and the exposure/dose level at which it first occurs. Eventually, more than one such endpoint needs to be considered, especially when particular toxic outcomes affect particular population groups as is the case with developmental toxicity.

When conducting hazard assessment, a distinction is often made between toxic effects that have a threshold of toxicity and those that show an effect at any

observable exposure level regardless of how low it is (nonthreshold effects). In the first case, it is assumed that the exposure must exceed a certain level before a toxic effect is manifested. Consequently, a "safe" maximal exposure can be calculated, below which damage is not likely to occur.

In the second case, it is assumed that an effect would occur at every exposure, be it so low. In such cases, the probability of a damage (e.g., 1 in 1,000,000) is often calculated for extremely low doses using a variety of mathematical models. Such an approach is mainly applied in the case of genotoxic carcinogens. Modern approaches to risk assessment suggest that such a differentiation is artificial and that all data should be treated in the same manner.

Hazard identification should, to the extent possible, include a description of the mode of action or, if data are available, the exact mechanism of action.

Usually, as the dose increases, the measured response also increases. At low doses, there may be no response. The adverse effect that occurs at the lowest dose is selected as the critical effect for risk assessment (e.g., Acute Reference Dose (ARfD), Lowest or No-Observed–Adverse-Effect-Level (LOAEL/ NOAEL), or ideally BMD limit (BMDL) see) which serves for the derivation of a health-based guidance value (Margin Of Exposure (MOE) or Tolerable Daily or Weekly Intake (TDI/TWI)) (Table 1).

The concept of an ADI was introduced in 1961 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Expressed in milligrams of additive per kilogram body weight, it is the dose, if consumed on a daily basis over a lifetime that would cause no adverse effects. The ADI/ TDI approach has been applied to toxic substances including non-genotoxic carcinogens. ADIs and TDIs are derived by dividing a surrogate for the threshold/points of departure by a standard uncertainty factor (UF) of a 100-fold (see paragraph below).

Table 1 Reference points and Health Based Guidance Values

Reference point (RPs)	Health-Based Guidance Value (HBGV)	Risk characterization
Benchmark Response (BMR)	Acceptable Daily Intake (ADI)	Margin of Exposure (MOE)
Lowest Benchmark Dose (BMDL)	Tolerable Daily Intake (TDI)	Risk Characterization Ratio (RC)
Benchmark Dose (BMD)	Acute Reference Dose (ARfD)	Hazard Quotient (HQ)
No Observed (Adverse) Effect Level (NO (A)EL)	Reference Dose (RfD)	Margin of Safety (MOS)
Lowest Observed (Adverse) Effect Level (LO(A)EL)	Derived-No-Effect-Level (DNEL)	Population at Risk
No Observed (Adverse) Effect Concentration (NO(A)EC)	Derived-Minimal-Effect-Level (DMEL)	
Lowest Observed (Adverse) Effect Concentration (LO(A)EC)	Population Adjusted Dose (PAD)	

According to EFSA, tolerable upper intake level (UL) is defined as the maximum level of total chronic daily intake of a nutrient (from all sources) judged to be unlikely to pose a risk of adverse health effects to humans. "Tolerable intake" in this context highlights what is physiologically tolerable and is a scientific judgment as determined by assessment of risk, i.e., the probability of an adverse effect occurring at some specified level of exposure. ULs may be derived for various life stage groups in the population. However, the UL is not a recommended level of intake. It is an estimate of the highest level of intake which carries no appreciable risk of adverse health effects. To establish whether an exposed population is at risk requires a risk assessment to determine what is the fraction (if any) of the population whose intake exceeds the UL and the magnitude and duration of the excessive intake (EFSA 2006).

The BMD is defined as a dose level, derived from the estimated dose–response curve, associated with a specified change in response, the Benchmark Response (BMR) (e.g., 0.1%, 1%, 5%, or 10% incidence). The BMDL is the lower confidence bound and is often used as the reference point, e.g., for a BMR of 5%, the BMDL05 can be interpreted as follows: a dose for which the response is likely to be smaller than 5% and for which where the term "likely" is defined by the statistical confidence level, usually 95% confidence (EFSA 2009; Muri et al. 2009) (Fig. 1).

Especially for acute toxicity, the acute reference dose approach (ARfD) as defined by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) is being applied to pesticides. The ARfD of a chemical is "an estimate of the amount of a substance in food and/or drinking water, normally expressed on a body weight basis, that can be ingested in a period of 24h or less without appreciable health risk to the consumer on the basis of all known facts at the time of the evaluation" (JMPR 2002).

Uncertainty Factors

The uncertainty factor of a 100-fold takes into account interspecies differences and human inter-individual variability. This UF allows for interspecies differences and human variability and has been subdivided to take into account toxicokinetics and toxicodynamics with even values of 100.5 (3.16) for the human aspect. Ultimately, such refinements allow for chemical-specific adjustment factors and physiologically based models to replace such uncertainty factors (Dorne 2010). Intermediate to chemical-specific adjustment factors are pathway-related uncertainty factors which have been derived for phase I, phase II metabolism and renal excretion. Pathway-related uncertainty factors are presented here as derived from the result of meta-analyses of toxicokinetic variability data in humans using therapeutic drugs metabolized by a single pathway in subgroups of the population. Pathway-related lognormal variability was derived for each metabolic route. The resulting pathway-related uncertainty factors showed that the current uncertainty factor for toxicokinetics (3.16) would not cover human variability for genetic polymorphism and age differences (neonates, children, the

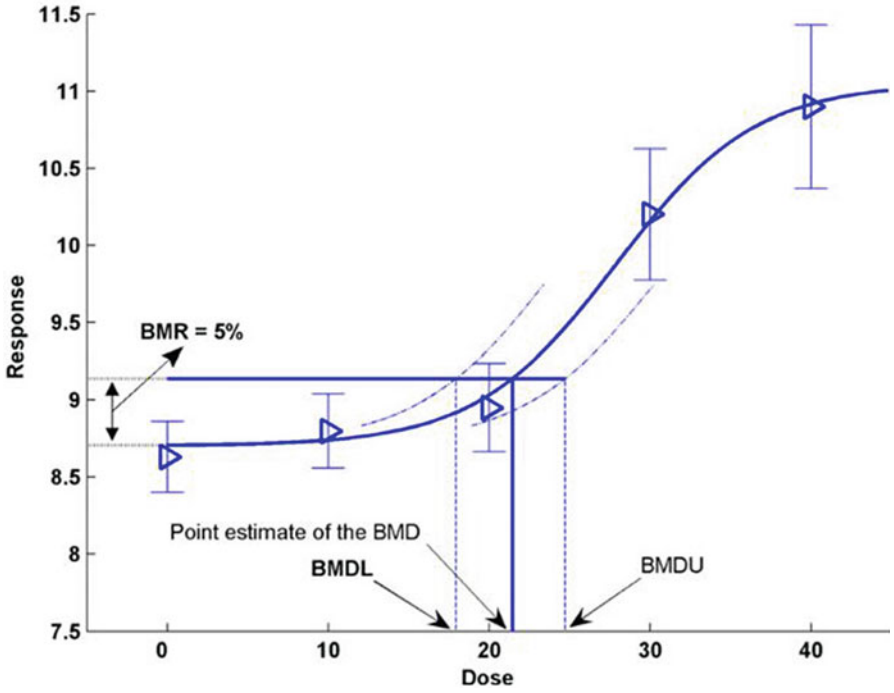

Fig. 1 Key concepts for the BMD approach, illustrated using hypothetical continuous data (EFSA 2017a). (The observed mean responses (triangles) are plotted, together with their confidence intervals. The solid curve is a fitted dose–response model. This curve determines the point estimate of the BMD, which is generally defined as a dose that corresponds to a low but measurable change in response, denoted the benchmark response (BMR). The dashed curves represent, respectively, the upper and lower 95% confidence bounds (one sided) for the effect size as a function of dose. Their intersections with the horizontal line are at the lower and upper bounds of the BMD, denoted BMDL and BMDU, respectively. It should be noted that the BMR is not defined as a change with regard to the observed mean background response, but with regard to the background response predicted by the fitted model. This distinction is important because, in general, the fitted curve does not hit the observed background response exactly (so that adding the BMR to the observed background response will in general not provide the correct intersection with the dose–response at the BMD). In the Figure, the BMD corresponds to a 5% change in response relative to background (BMR = 5%). The fitted curve yields an estimated background response of 8.7, and a 5% increase of that equals 9.14 (= 8.7 + 0.05 × 8.7). Thus, the BMD05 of 21.50 is obtained from the intersection of the horizontal line, at a response of 9.14, with the fitted dose–response model. In this example, the BMDL05 has a value of 18)

elderly). Latin hypercube (Monte Carlo) models have also been developed using quantitative metabolism data and pathway-related lognormal variability to predict toxicokinetics variability and uncertainty factors for compounds handled by several metabolic routes. For each compound, model results gave accurate predictions compared to published data and observed differences arose from data limitations, inconsistencies between published studies, and assumptions

during model design and sampling (Dorne 2010). Ideally, chemical-specific adjustment factors (CSAFs) derived from physiologically based toxicokinetic–toxicodynamic models are established (e.g., assessment of cadmium) (Amzal et al. 2009; EFSA 2009).

Other Approaches

When a quantitative dose-response relationship cannot be defined, a semiquantitative or qualitative analysis will have to be done. More specifically, in the absence of adequate human and animal toxicological data, structure-activity relationship data can also provide useful insights into the prediction of toxicokinetics of the chemical in the body and an indication of its toxicity for groups of structurally related chemicals or individual chemicals and can include the threshold of toxicological (TTC) approach (EFSA and WHO 2016).

The principle of Threshold of Toxicological Concern (TTC) has been developed and is now used by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in their evaluations. Establishing an accepted TTC would benefit consumers, industry, and regulators, since it would preclude extensive toxicity evaluations when human intakes are below such threshold, and direct considerable time and cost resources towards testing substances with the highest potential risk to human health (Kroes et al. 2000).

Exposure Characterization

As the Swiss physician Paracelsus stated in the sixteenth century, "all substances are poisons; there is none which is not a poison. It is the dose which determines a poison."

In characterizing exposure, it is important to start by describing the applied exposure assessment methods, as well as their strengths and weaknesses. If different approaches to exposure assessment were employed in parallel, a comparative assessment of all of them should be presented.

Exposure characterization should include a description of all exposure sources and the contribution by all relevant environmental media (air, water, food, soil) to the total exposure. While the total exposure must be determined, it may be important under certain conditions to consider exposures related to different routes (e.g., inhalation and ingestion) separately. This is particularly important in cases where different toxicological effects are observed following exposure via different routes. Furthermore, it is important to consider all environmental compartments that lead to an exposure (such as the workplace or the general environment) separately when determining and describing the overall exposure.

Finally, it is crucial to determine the exposure of particularly vulnerable groups of the population and to identify all such groups that are subject to higher exposure levels.

Uncertainty and Variability

Although, ideally risk assessments would be based on a more than adequate knowledge base, in reality it is not always the case. This means that all risk estimates are uncertain to some degree. To address this, the risk assessor should always consider the uncertainties and variabilities in the calculations.

In the process of risk assessment, uncertainties may stem from the lack of data or because extrapolations are necessary. These must be identified in the risk characterization. In addition, variability aspects must be fully considered, be they between individuals or between particular groups of the population. A good risk characterization will address and describe both aspects.

Questions related solely to uncertainty are those that address a lack of knowledge or information. Examples include the eventual need (due to lack of data) for extrapolation from short- to longer-term exposures (e.g., from subchronic to chronic exposure), the extrapolation from lowest dose that results in an adverse effect (lowest observed [adverse] effect level, LO[A]EL) to the highest dose that shows no adverse effect (no observed [adverse] effect level, NO[A]EL). This category of pure uncertainty aspects also includes all deficiencies of the database, for example, the lack of certain studies. Such studies could be ones linked to certain exposure duration or ones that assess certain toxic endpoints (e.g., studies on neurotoxic or reproductive effects). In such instances, it is critical to evaluate to what extent the missing information could change the overall assessment and to provide a clear and logical description of such an evaluation. This requires appropriate experience and often a knowledge of the mechanism of action, besides information on exposure and toxicokinetics. Generally, with increasing information the certainty of the risk assessment increases as well (Fig. 2).

The dose that causes an effect can vary from one person to the next depending on factors such as genetic differences, preexisting medical conditions, etc. Similarly, Exposure varies from one person to the next depending on factors such as working

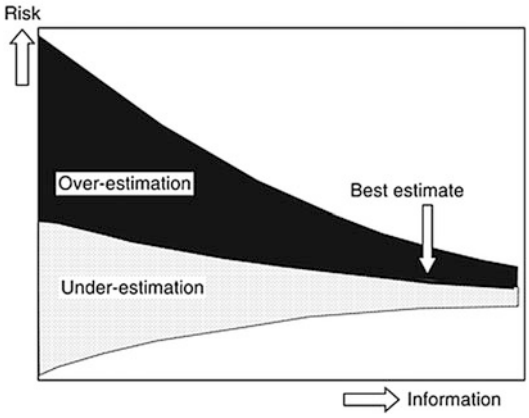

Fig. 2 Precision of a risk estimate as a function of available information. The more data are available, the lower the uncertainty around the risk estimate

conditions, life conditions, geographical food habits, etc. Questions related to both uncertainty and variability are encountered in cases where interspecies extrapolation is necessary. This is the case, for example, when the health risk to humans is evaluated based on data from animal experiments. The variability between individuals in a given population group (intraspecies variability) also plays an important role. In both cases, the variability encompasses aspects of toxicokinetics and toxicodynamics, including, inter alia, the contact rate, uptake or absorption, general systemic availability, systemic elimination, active site concentration, physiological parameter changes at site of effect, and the functional reserve capacity. Aspects of variability must all be fully considered in the risk characterization process.

When assessing the potential for risks to people, toxicology studies generally involve dosing of test animals as a surrogate for humans. Since we don't really know how differently humans and rats respond, EFSA and other international organizations often use an uncertainty factor to account for the aforementioned differences (interspecies differences). Additional consideration may also be made for differences within the tested animals such as the sex, the age, etc. (intraspecies differences).

In certain cases, special issues may play a role in risk characterization. In the case of persistent compounds that bioaccumulate, such as PCBs, dioxins, and persistent chlorinated pesticides, risk characterization should rather be based on the total body levels over exposure time (body burden) rather than on the external exposure or a dose over a limited period of time. With these compounds, it is also important to consider that an exposure in utero is not only related to an eventual exposure during pregnancy, but rather on the body burden of the mother at that time. Another example is that of substances that show cumulative effects, such as cholinesterase inhibitors, which exert their toxic effects through a common point of action.

Weight of Evidence

When the scientific question is relatively simple and can be addressed directly, then a straightforward assessment with the steps described in this charter can be conducted to reach an outcome.

In many assessments, however, questions may need to be subdivided to yield more directly answerable questions and a weight of evidence assessment needs to be conducted (see ▶ Chap. 6, "Toxicological Risk Assessment"). The weight of evidence has three core steps: Assembling the evidence, weighing the evidence, and weighing the relative support for possible answers to the question (after EFSA 2017b).

Transparency of the Process

In risk characterization, the whole process of risk assessment must be clearly outlined, and all elements necessary for decision-making on managing potential risks sufficiently

described. In this respect, information on the scope of the risk assessment (why was it conducted?) on the extent and quality of the database, as well as on the date of the last literature search were conducted. Furthermore, it must be stated if, and if yes why, adjustment or uncertainty factors were used. Finally, all elements of the decision process must be described, including the mechanism of peer review.

Cross-References

- ▶ Risk Evaluation in Regulatory Toxicology
- ▶ Toxicological Risk Assessment

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Risk Evaluation in Regulatory Toxicology

57

Maged Younes and Nikolaos Georgiadis

Contents

Abstract

Risk evaluation is the step within the risk analysis process that links risk assessment (the final step of which is risk characterization) with risk management. This intermediary step is mostly not explicitly mentioned, or it is seen as a preliminary step in risk management. The goal of risk evaluation is to link exposure levels with corresponding risks and to identify sources of uncertainty in the scientific data used.

M. Younes (✉)
Global Publich Health, Mies, Switzerland

Formerly: Food Safety and Zoonoses, formerly: World Health Organization (WHO), Mies, Genève, Switzerland

N. Georgiadis
Toxicologist, European Chemicals Agency (ECHA), Helsinki, Finland
e-mail: nikolaos.georgiadis@echa.europa.eu; nikolaos.georgiadis@gmx.com

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Keywords

Risk acceptance · Risk assessment · Risk evaluation · Risk-management options · Risk perception · Socio-economic considerations

Introduction

Based on the outcome(s) of the risk assessment, options for risk management need to be developed and evaluated for the decision-making process. Risk management covers all actions and decisions as to whether or not, and, if yes, how certain risks should be eliminated or reduced. Options and strategies developed for their possible implementation can be of regulatory, economic, informational, or technological nature. They need not to be mutually exclusive. In order to reach adequate and rational decisions, a risk must be seen in the context of other risks and evaluated considering various different factors.

Good risk management decisions should follow certain principles. Thus, the problem must be discussed within its health or ecological context and should consider the views of all those who would be affected by possible decisions. Such decisions must be based on a balanced scientific assessment and build on a full analysis of different regulatory and nonregulatory options for action. They must lead to a reduction or an elimination of the risk under consideration and be implementable in a rapid and efficient manner and with the support of all relevant stakeholders. Actions must, indeed, be proven to affect the risk to be minimized or eliminated. They should offer the possibility for being revised or changed if new information becomes available that would justify it.

Elements of Risk Evaluation

To allow for sound risk management decisions to be reached, risk evaluation should offer ways to eliminate or reduce the risk(s) under consideration that fulfill certain criteria. Risk evaluation should be based on scientific, technical, and economic data of the highest possible quality. It should take account of the mostly existing context of multiple risks. Recommended actions must be technically, politically, and economically feasible and should offer clear advantages with regard to cost. They should give preference to prevention and place innovation in the center of the decision-making process. Finally, they should take account of sociopolitical aspects. Some elements of risk evaluation are discussed in more detail in other chapters of this book. In this chapter, the main aspects of risk evaluation will be briefly discussed and relevant terms will be explained (Fig. 1).

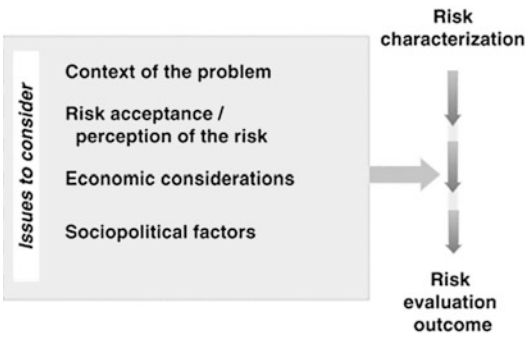

Fig. 1 Issues to consider within the framework of the risk evaluation process

Context of the Risk Problem

A risk cannot be evaluated in an isolated manner. Often, a risk factor, for example, a chemical substance, has several sources that contribute to the overall exposure. In such cases the risk should be evaluated within the "multiple source context." The risk evaluation must consider the contribution by each of these sources to the overall exposure in order to permit the development of effective risk management options that will, indeed, reduce the risk. The question here is mostly about the point of intervention that would achieve the most effective protection.

An exposure to the same risk factor could occur through different environmental media (e.g., air, water, drinking water). One example is lead. Human exposure is via air (inhalation; the main sources here are car exhaust emissions if lead-containing petrol is used, lead-containing paint, and various industrial processes), via drinking water (ingestion, e.g., in the case of lead water pipes), via food (ingestion, mainly through the use of lead-containing food cans), as well as via direct exposure through the use of lead-containing cosmetics (e.g., dermal exposure). A risk evaluation must therefore also consider exposure through all relevant environmental media ("multi-media context").

The source of one risk factor might also release other substances that may pose an additional risk. Dioxins, for example, are encountered as food contaminants (e.g., in fish), often in combination with, inter alia, polychlorinated biphenyls, and methyl mercury. The three compounds have all neurotoxic effects among other toxic actions. The combined effects must be considered jointly. In such cases, individual compounds should not be evaluated independently from accompanying exposures ("multifactor context").

Finally, a risk must be evaluated in comparison to other risks in the same group of population. This is important in order to set priorities for action and to initiate actions which are most urgently needed first ("multiple risk context").

Risk Acceptance

The decision as to whether or not a risk is acceptable requires a judgment in the context of social, political, and economic aspects. Of special importance to the risk evaluation is the way society judges the particular risk under consideration and to what extent certain exposures would be tolerated. Risk acceptance depends to a large extent on the perception of risk. Risks are not always seen by the public in scientific terms but often also based on qualitative perception. Thus, risks are accepted if they are common and known, if they are easy to control, if their mode of action is known, if the exposure is voluntary, if the effects are immediately seen and do not affect future generations, and if potential effects are reversible and/or are not of catastrophic nature. Trust in responsible institutions, lack of media interest, and clearly visible economic benefits also increase risk acceptance. In general, risks are more accepted if they are easy to see and their control appears to be easily accessible. Thus, the risk of a nuclear reactor accident is judged to be higher than that of a motorcycle accident or that of smoking. Aspects of risk acceptance must therefore be clearly delineated and fully considered in risk evaluation. Risk comparisons may help in providing an objective view in this context.

Socio-economic Analysis

Socio-economic analysis is a well-established method of weighing up the pros and cons of an action for society as a whole and need to contain a description of the risks as well as information on the health and environmental benefits, the associated costs, and other socio-economic impacts. In asocio-economic analysis, one needs to analyze and document whether the socio-economic benefits of continued use of the substance outweigh the risks of continued use for human health and the environment.

Economic Factors

An economic valuation provides important information for risk management. Economic considerations should therefore form an integral part of risk evaluation, including potential benefits which would be brought about through an improved health and environmental protection. In particular, two aspects should be looked at. The cost-benefit analysis considers economic and/or social gains emanating from a risk-producing process in comparison to its costs, which should also include those costs related to eventual damages to human health and environmental integrity. The cost-effectiveness analysis, in contrast, evaluates rather the "efficiency of a certain intervention" (e.g., a regulatory measure or a technological evaluation) in controlling a certain risk. Here, the expected economic and/or social benefit due to a certain proposed measure is quantified and compared to

the cost caused by such a measure. In both processes, the quantification of positive and negative effects on human health and the environment in economic/monetary terms is a major problem.

Sociopolitical Factors

Risk management decisions are political in nature. Therefore, options developed in risk evaluation need to reflect social and political considerations. Among the questions to be addressed, the issue of other risks that occur concomitantly and need also to be managed figures prominently. In this context, different risks are evaluated in a comparative manner, and a rational weighting is performed. Often, it is necessary to assess which risks should be given priority in reaching risk management decisions and which can be addressed at a later stage, since it is difficult, if not impossible, to address all risk factors at the same time. This process of comparing risks and weighing risk management options is defined as "risk balancing."

Sometimes, an action taken to control a given risk factor may lead to the appearance of new risks to health and/or the environment. Replacement of a chemical in a technological process with another, for example, can produce new risks. A practical example would be to abandon the disinfection of drinking water to avoid the risk due to chlorination by-products: the expected reduction of the health risks due to chlorinated organic compounds in drinking water would be linked to a significant increase in the risk of waterborne infectious diseases. Considerations of this kind fall under the term "risk-risk tradeoffs." The main question to be addressed here is: "which risks do we take if we control another through certain measures?"

Another aspect of sociopolitical and ethical nature is that of "environmental equity." In this context, considerations are made as to whether or not the population group(s) that benefits from a certain activity is the same as those who carry the risk. The aim is to avoid situations where a group carries all (or the larger part) of risk, without profiting from the risk-producing process, and that all benefits come to another group that carries no or a substantively lower part of the risk.

Uncertainty and Variability: Scientific and Economic Aspects

Since risk evaluation is an intermediary step linking risk characterization with risk management, uncertainty and variability issues that emerged and were discussed in risk characterization must also be fully considered when developing options to minimize or eliminate risks. Problems that could be of relevance at this step could target scientific or economic aspects. Examples of scientific issues include the relevance of toxicological studies under real-life conditions (risk prediction), the possibility to detect and consider differences in susceptibility among exposed populations, as well as the identification of highly exposed groups. In addition, questions concerning realistic exposure scenarios and on interactions between different risk factors may be of relevance. Economic problems include the difficulty of

quantifying health and environmental aspects from an economic point of view. Other issues target the inconsistency of economic analyses and the uncertainties connected with it, as well as the inadequacy of methods to validate the advantages of potential risk management actions for human health and the environment.

Outlook

Aspects described in this chapter are within the context of evaluating a given risk in the context of other risks and with consideration given to risk acceptance, as well as political and economic factors. Such considerations constitute a judgment of the characterized risk in connection with the development of control options as a prerequisite for managing the risk. Risk evaluation, thus, has a bridging function between the pure science and the political decision-making process. Even though this step is not explicitly included as a separate process in the usual risk assessment and risk management paradigms, risk evaluation is an important basis for decisions. Modern approaches to risk analysis, such as the one proposed by the US National Research Council in 2009, promote a more integrated approach to risk assessment and management, during which questions related to risk evaluation are addressed from start (problem formulation) to end (risk management), ensuring stakeholder involvement and risk communication throughout.

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▶ Risk Characterization in Regulatory Toxicology
▶ Toxicological Risk Assessment

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Risk Comparison in Toxicology

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Franz-Xaver Reichl

Contents

Abstract

Statisticians have calculated probabilities for most of the circumstances of everyday life, including the chances that an individual will become ill, have an accident, or die. The danger profile for a single individual is divided into a multitude of individual risks, which are unequally distributed, sometimes starting from birth. For example, 2% of all diseases are genetically determined. Even people who arrive in the world healthy, however, have disadvantages, but factors such as success in an occupation and high income are protective against early death. Statistically high risks are associated with smoking and poor nutrition, whereas the risk of death from viruses, radiation, or chemicals is low.

The calculation of risks is difficult and dangers often arise. Experts as well as laypeople may fall victim to "cognitive dissonance," where knowledge that disturbs established beliefs and habits is not perceived. Risk assessment thus becomes difficult, as seen by the following phenomena:

F.-X. Reichl (✉)
Department of Conservative Dentistry and Periodontology, University Hospital, LMU Munich, Germany and Walther-Straub-Institute of Pharmacology and Toxicology, LMU Munich, Munich, Germany
e-mail: reichl@lmu.de

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- The occurrence of rare hazards (e.g., snake bite) is overestimated, whereas that of frequent hazards (e.g., death caused by cardiac infarct resulting from obesity) is underestimated.
- People are subject to an imperfect assessment of probabilities; for example, driving in a car is more dangerous than flying in a plane, but the fear of flying is common compared with the fear of driving.
- People tend to overestimate their own abilities (e.g., they believe they are immune from disease).
- Fears are minimized for things people believe they can control (e.g., smoking).
- Fears are maximized for things people believe they cannot control (e.g., a toxic waste repository).

Psychological studies show that events with a high "horror factor" (e.g., being eaten by a shark) are particularly feared, even if they occur extremely rarely. Human behavior is less determined by numbers and facts than by faith, desires, and fears.

Keywords

Absolute risk · Relative risk · Statistics · Life expectancy · Mortality · Morbidity · Fears · Risk assessment · Risk management · Cognitive dissonance · Natural catastrophes · Risk acceptance · Risk characterization · Risk profile · Risk appraisal

Introduction

Insurance companies and security specialists define risk as the product of probability of occurrence and level of damage, and calculate premiums accordingly. So, the risk remains the same, regardless of whether minor damage occurs frequently or major damage occasionally. According to this principle the risk for 1,000 road accidents, each with one adult killed, is exactly the same as that of a school fire in which 1,000 children die. In general, however, the word "risk" encompasses both danger and chance. It describes both the objective threat which cannot be avoided and also the subjective gamble which is assumed voluntarily. A danger survived can therefore also become a chance for a better and safer life.

The idea that each person holds his fate in his or her own hand did not emerge until after the Middle Ages. While prior to this many people believed in evil or good-natured gods, who at least partly determined fate, in modern times each individual rose to become important producers of dangers and chances. And because at that time, there was no term for this concept, a new word had to be coined. The work "risk" derived etymologically in the sixteenth century from the Italian word *risco* (gamble, hazard), which was in turn probably derived from the Greek *rhiza* (root; secondary meaning: cliff) or from the Arabic *rizq* (livelihood which depends on God and fate).

Definitions

- *Absolute risk* in an equally affected group of persons is the ratio of the number of illnesses to the total number of persons.
- *Relative risk* is the ratio of the absolute risk of the affected group to the unaffected group.
- *Risk appraisal* (risk description) is the quantitative determination of possible health risks due to chemicals or radiation depending on efficacy, length of exposure, and level of exposure or the dose absorbed.
- *Risk assessment* is the evaluation of a risk with regard to its tolerability under social and health political aspects.

Risk Structures

Statisticians have calculated the probabilities for all possible circumstances of everyday life that an individual will, e.g., contract an illness, have an accident, or die. Their calculations of the risk of illness ("morbidity") and the risk of dying ("mortality") are, however, applicable only to an imaginary being: the statistical average person.

His existence follows a risk profile which changes dramatically with age. Already on the first day after birth, one in every 600 newborns dies in Germany, as the result of, for instance, having too low a birth weight or pregnancy complications (Cawley et al. 2020). In the first year of life, the rate increases to one in 125 babies. After the first birthday, survival odds then rise steeply. Ten years after birth and survival of childhood diseases, the safest phase of life is reached. The annual risk of death reduces to the lowest level of 1 in 6,000, before increasing again between the ages of 15 and 20 for the average male teenager to 1 in 1,000 due to the propensity to take risks (e.g., driving). In addition, the willingness to take their own lives increases: Around one in five men who die around the age of 30 commit suicide. Diseases (e.g., cardiac and circulatory diseases, cancer) (Samet et al. 2020), which are the cause of death for 95% of people, only dominate from the age of 40, and in the decade before retirement bring the mortality rate back up to the level of infants.

This basic risk structure has not changed for thousands of years. Already in the Paleolithic Age, death claimed mainly infants and old people and granted security in late childhood. Equally, young people took risks, although in those times not on the roads but perhaps in hunting.

But any attempt to derive one's personal destiny from these figures is senseless. The **risk profile** for the individual is made up of millions of individual risks which are often unequally distributed right from birth. Two percent of all diseases are, for instance, genetically determined. And a child born healthy but male already has a disadvantage. In their very first year of life, around one third more boys die than girls. Women are less likely to commit suicide and do not have as many accidents on the road or at work. They also used to drink and smoke less than men – an advantage which is, however, diminishing in the name of emancipation.

Professional success, high income, and a good education are on the other hand factors which protect men in particular against an early death. According to an US study, rich men, for instance, in Canada live four-and-a-half years longer than the poorest ones there. Background, poverty, poor living conditions, unemployment, and contaminated food (de Boer 2019) reduce **life expectancy**. This becomes particularly clear in the New York district of Harlem which is populated almost entirely by non-whites, almost 50% of whom live below the poverty line. In this area, mortality rates in women between 25 and 34 and in men between 35 and 44 are six times higher than the US average. The likelihood of reaching the age of 65 in Harlem is less than it is in Bangladesh.

Other various risks also contribute to reducing life expectancy, e.g., the life expectancy of men who smoke cigarettes reduces on average by almost 7 years (Fig. 1). One way to reduce personal risk slightly (on a purely statistical basis) is a trip to the registry office. But whether marriage really offers more security is not certain. Perhaps single people between the ages of 35 and 45 have a riskier way of life. Divorce and even more so the death of a partner drive many to an early grave – although women cope with the loss considerably better.

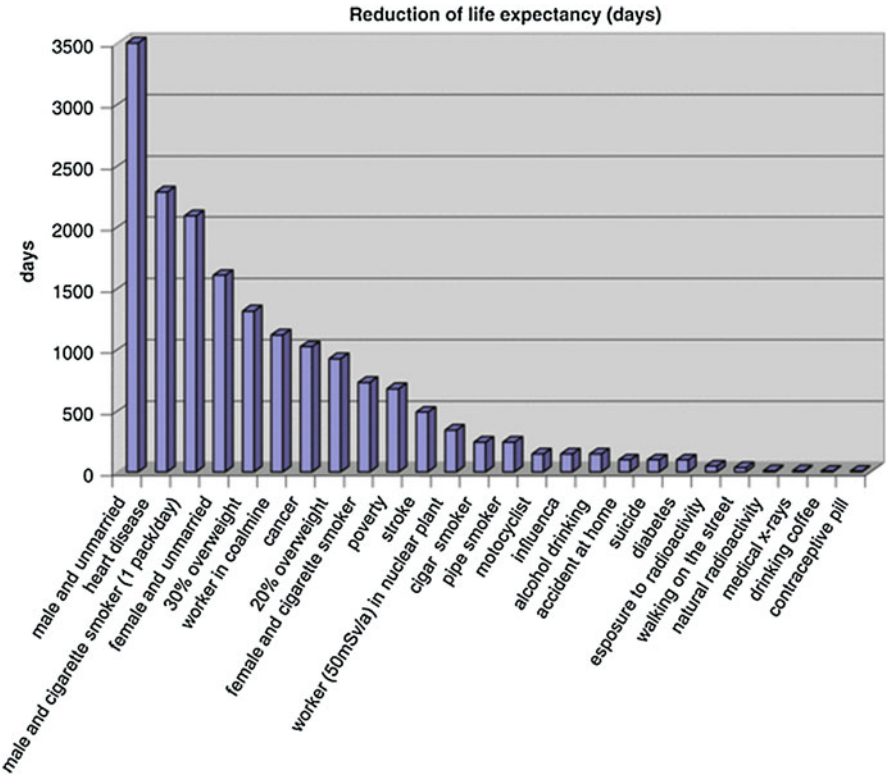

Fig. 1 Reduction of life expectancy through various risks in the population of Europe

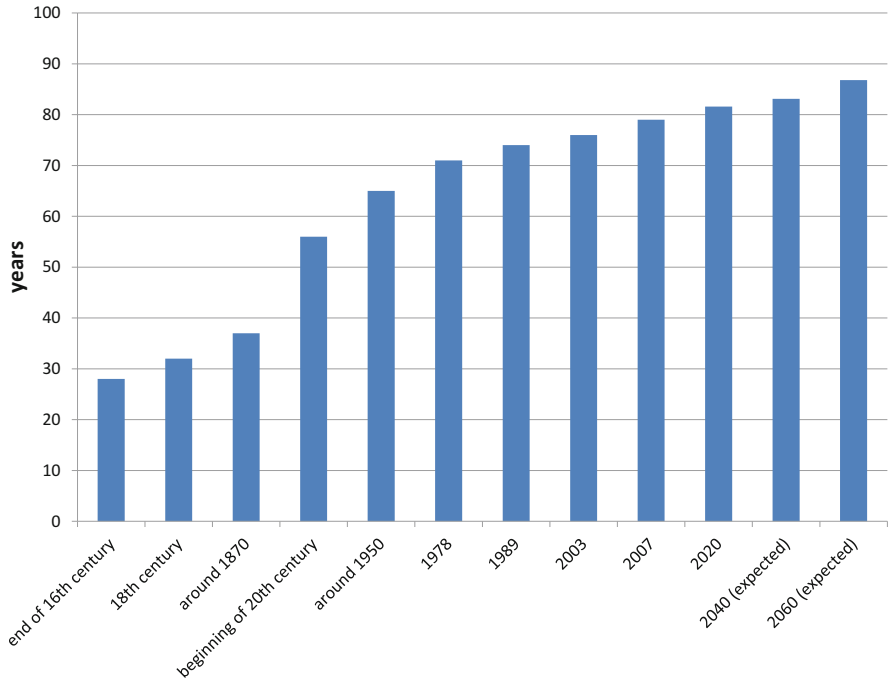

Fig. 2 Development of life expectancy in Germany (years; average of men and women)

Today, statistically speaking, Germans are getting around three times as old as they were 300 years ago at 85 years (women) and 79 years (men) (Fig. 2). The main reason for this is the decline of infant mortality. For the year 2040, in Germany, a life expectancy of 86 years (women) and 81 years (men) and for 2060 89 (women) and 85 (men) is forecast (Fig. 2). The life expectancy of persons in Afghanistan has up to now not got beyond 45. Almost all over the world, women live longer than men, with the exception of countries such as Afghanistan or Bangladesh, in which they are severely disadvantaged (Fig. 3).

Future Risks

Compared to smoking and nutrition, today, **radiation** and **chemicals** (still) represent relatively minor risks. In the future, however, four risks from the environment and biology could be a particular threat:

- Damaging ultraviolet (UV) radiation: according to US calculations by 2100, skin cancer will claim the lives of an additional 10,000 people as a result of increased UV radiation on the Earth due to the reduced (protective) ozone layer.
- In the USA, it is estimated that passive smoking causes up to 8,000 deaths a year.

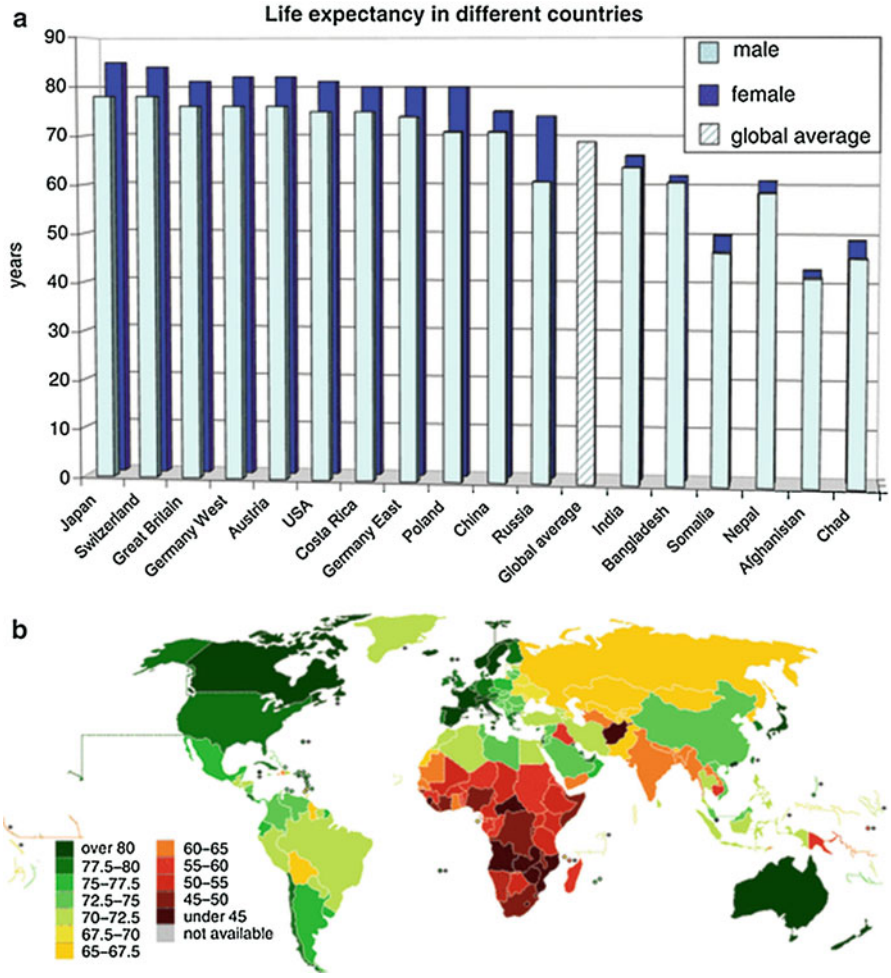

Fig. 3 (**a**) Life expectancy in various countries. (**b**) World map indicating Life expectancy at birth in UN member states

- The radioactive gas radon, which comes up through the ground and penetrates natural building materials, today also already claims the lives of around 20,000 Americans.
- Viruses may kill thousands of people worldwide and may cause long-term economic damages of trillions US-dollars as already happened for the virus SARS-Covid2 in the year 2020.

The risk of contracting cancer from the more than 60,000 chemicals is relatively low. According to the most recent studies, this contributes a total of only around 1–2% of the overall cancer risk to humans. However, 50,000 materials have still not even been tested

for their carcinogenic potential. It is still disputed just how dangerous these over 600 substances are which have proved carcinogenic in animal experiments. What induces cancer in a rat may have no effect on a mouse and vice versa.

Another problem still unresolved is the effect of carcinogens in combination. The effect of radon contaminated inside air, asbestos, or alcohol must not simply be added to the risk factor of smoking. In combination, these substances may even exponentiate the risk of lung cancer.

Patients are also increasingly complaining of the onset of symptoms after a tooth restoration, because they believe they are being slowly poisoned by the substances released from the inserted dental materials in combination with other (environmental) pollutants. What risk actually exists for these patients can be established by a recognized international toxicological dental advice center (e.g., at the Ludwig-Maximilian University of Munich: www.dentaltox.com).

Because the analysis of health risks is fraught with many uncertainties, statisticians tend to resort to averages calculated from many individual findings. This does, however, have its pitfalls: Risks which are meaningless for the general public can be a major threat to those in certain risk groups, e.g., children who sit in classrooms contaminated with asbestos. Although in the average population of the USA, 100 times more schoolchildren die at football games than from asbestos poisoning, anyone who is exposed to the deadly dust at relevant concentrations bears a high individual risk.

Comparisons such as "asbestos versus football" are intended to put risks into perspective. But one risk is not like any other, and not every relation is useful. This becomes particularly clear by considering, for instance, the correlation between the reduction of breeding pairs of storks in Germany and the reduction of the birth rate in Germany in the years 1965–1980 (Fig. 4): An accurate correlation, but not a useful one!

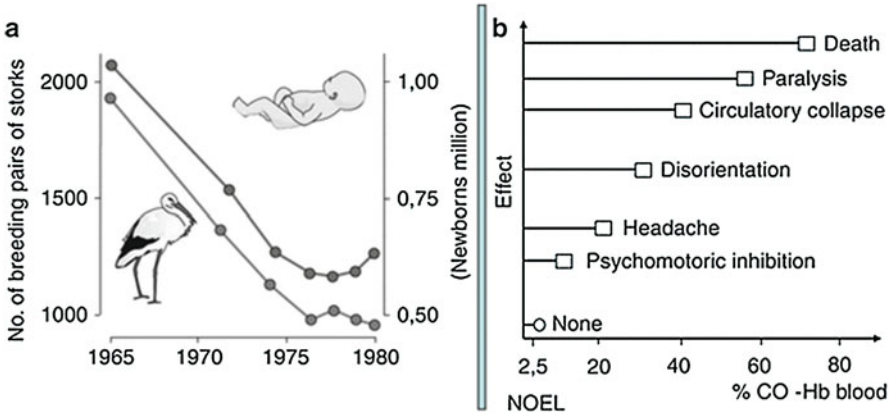

Fig. 4 Useful and senseless correlations. (**a**) Statistically significant correlation, but no causal link: decline of breeding pairs of storks and decline of birth rate in the Federal Republic of Germany from 1965 to 1980. (**b**) Statistically significant correlation, causal link: effect of an increasing carbon monoxide content in the blood

Mortality Risks in the Population

The calculation of risks is very difficult and often involves dangers. Experts are, namely, just as susceptible as lay people to what is known as **cognitive dissonance** (Reichl and Ritter 2011). The phenomenon by which knowledge which disrupts long-established habits is simply not perceived. Thus, for instance, in the USA in 1985, NASA published a risk assessment on the Space Shuttle, according to which the probability of crash for the shuttle was 1: 100,000. Studies by other committees, however, assumed a risk ranging from 1: 270 to 1: 57. In fact, the shuttle "Challenger" exploded on its 25th flight and the shuttle "Columbia" broke up on its 28th flight.

According to more recent studies, being killed by lightening is around 650 times more unlikely than dying as a consequence of alcohol – but dying as a result of cigarette smoking is almost 10 times more likely (Fig. 5). The risk of being killed by a meteorite crash is in fact greater than that of dying in a plane crash. Although the likelihood of being hit by a celestial body is astronomically small, if it did happen millions of people could die – so the result is an increased risk.

The probability for the average citizen of dying in a terrorist attack or a natural disaster is infinitesimal. It remains infinitesimal even though the number of, e.g., major natural disasters has been increasing globally since 1980 (Fig. 6).

It is this increase which is often the reason for escalating fear in the population, although in fact this is not justified. But escalating fears would be justified if you consider the indirect consequences of terror attacks or natural disasters, which affect every one of us, i.e., even the unaffected average citizen, and could thus precipitate

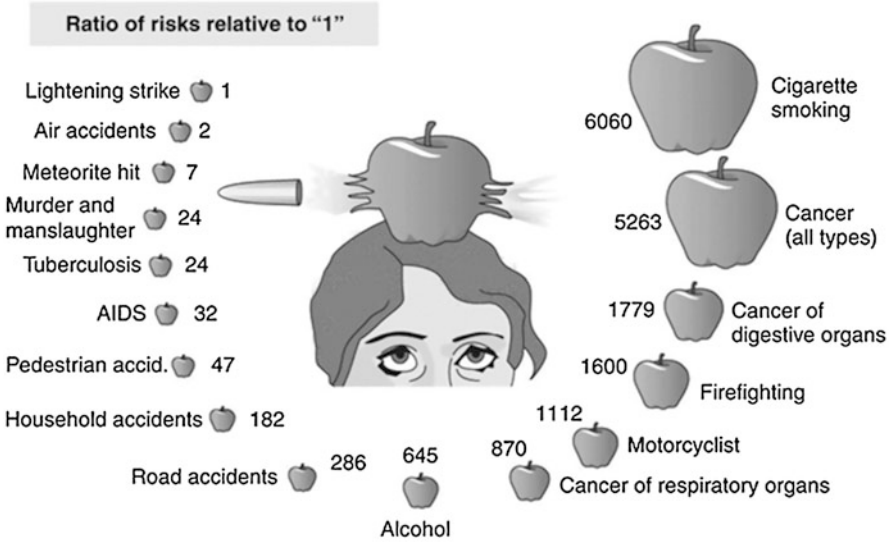

Fig. 5 Mortality risks in the population of Europe

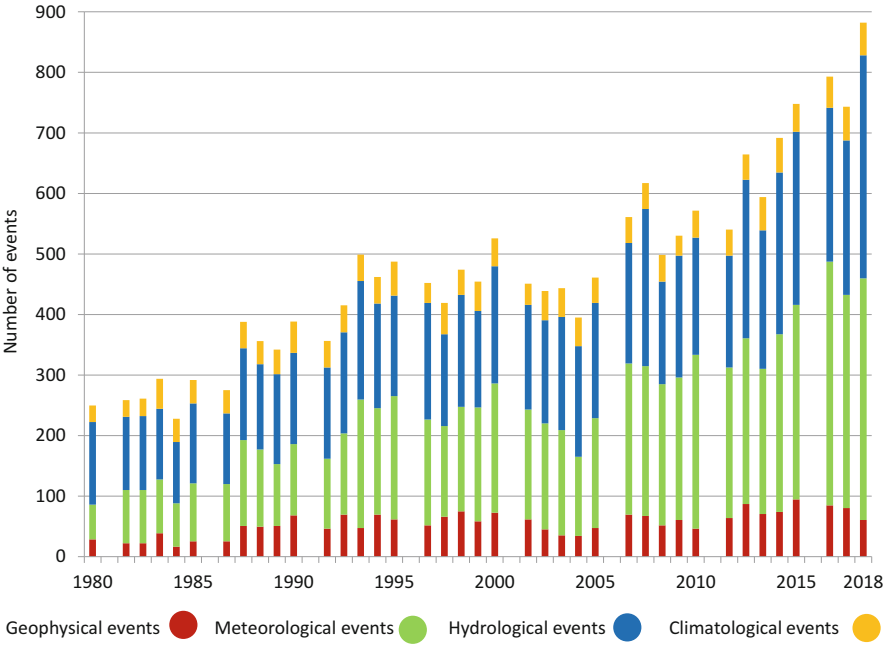

Fig. 6 Number of natural catastrophes. Number of natural catastrophes from 1980 to 2018 (worldwide; source: Münchener Rückversicherungs-Gesellschaft/GeoRisikoForschung, NatCatSERVICE 2020). **Geophysical events**: General terms having their origin in the earth crust like earthquake, tsunami, volcanic activity, and mass movement dry. **Meteorological events**: General term for loss events caused by all kinds of storms such as tropical storm, winter storm, convective storm, and local windstorm. **Hydrological events**: General term for loss events covering all kinds of floods and rain triggered mass movements (Tsunami is not included). **Climatological events**: General term for loss events covering all kinds of extreme temperature, drought, and wild fire

his downfall. Because the national economic damage caused by disasters has in fact increased dramatically over the last 60 years (Table 1), bringing some countries to the brink of ruin and even causing some reinsurance companies to topple (Fig. 7) (just the terrorist attack on the World Trade Center on 11 September 2001 caused national economic damage of almost half a billion Euros). The loss of immense sums of money (Fig. 7) means that there is then no longer enough funding available for necessary projects. Whole population groups can thus be thrust suddenly into economic poverty, which is in turn a factor for a shorter life.

Acceptance of Risks

The risk acceptance in the population is a complex and unpredictable phenomenon.

1. Rare risks (e.g., a snake bite) are overestimated, common threats (e.g., fatal heart attack due to excess weight and lack of exercise) are underestimated.

Table 1 Comparisons across decades of the number of major natural disasters occurring, the damage to the national economy, and the insured damage in billions of US dollars (worldwide; values from 2006; source: Münchener Rückversicherungs-Gesellschaft/GeoRisikoForschung, NatCatSERVICE 2020)

Major natural catastrophes 1950 -2006

	Dekade 1950-1959	Dekade 1960-1969	Dekade 1970-1979	Dekade 1980-1989	Dekade 1990-1999	last 10 1997-2006
Number	21	27	47	63	91	51
Overall damage	51,4	89,5	155,2	252,0	742,9	550,7
Insured damage	1,6	7,3	14,9	30,5	137,7	180,2

Comparison of the last 10 years to the 1960s shows the dramatic increase

Factor last 10:60s
1,9
6,2
24,7

• Damage in billions of US$ – values from 2006

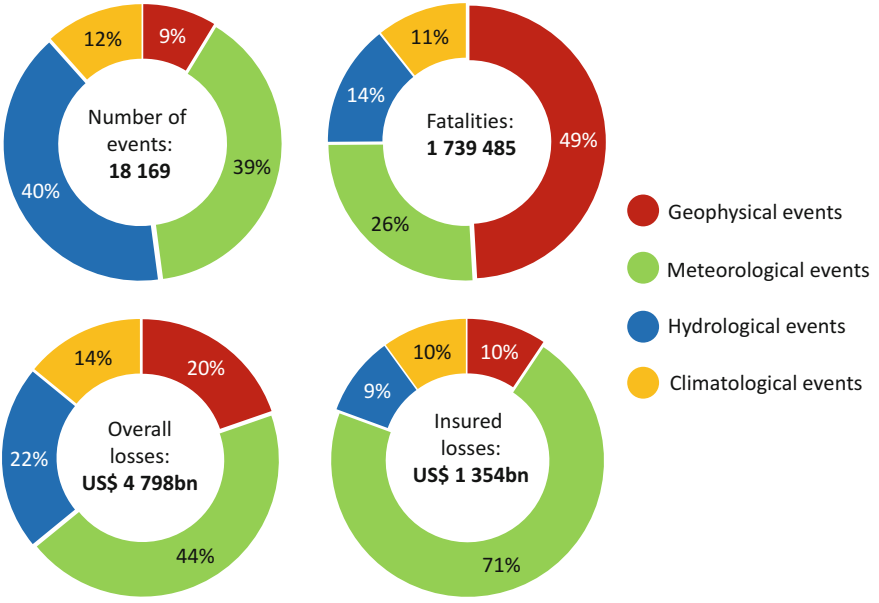

Fig. 7 Percentage distribution for relevant natural loss events worldwide from 1980 to 2018 (worldwide; source: Münchener Rückversicherungs-Gesellschaft/GeoRisikoForschung, NatCatSERVICE 2020). Explanation to the types of events: See legend to Fig. 6

2. People are liable to falsely evaluate probabilities (e.g., driving a car is more dangerous than flying, but we always talk about a fear of flying and never a fear of driving). People do not worry because the possibility of dying in each individual car journey is one in four million and thus lower than the chance of having a fatal

accident when mowing the lawn. But if you consider that in the course of your life you undertake several 1000 car journeys, the actual risk increases. One in 100 Germans dies as the result of a road traffic accident.
3. People overestimate their own abilities (e.g., they believe they are immune to disease or will have a long life).
4. Things which we believe are under our control minimize fear (e.g., smoking, drinking alcohol, driving, or climbing).
5. Things which we obviously cannot control ourselves increase fear (e.g., toxic waste facilities, invisible toxins in foodstuffs, atomic power plants).

Psychological studies show that events with a high "gruesomeness factor" (e.g., plane crashes, death by lightening, or being eaten by a shark) are especially feared, even though they are extremely rare. This is made particularly clear by the following study: When asked to choose between two forms of treatment, patients, and even doctors, preferred the treatment with a 90% chance of survival over that with a 10% mortality rate, although both figures express exactly the same thing: one in ten dies.

Thus, human behavior is steered less by facts and figures than by beliefs, desires, and anxieties. That is why in the future too, acceptance of risks will remain a fascinating and unpredictable social psychological phenomenon for us all.

Cross-References

- ▶ Risk-Benefit Considerations in Toxicology
- ▶ Risk Characterization in Regulatory Toxicology
- ▶ Risk Cycles in Toxicology
- ▶ Risk Evaluation in Regulatory Toxicology
- ▶ Toxicological Risk Assessment
- ▶ Toxicological Risk Assessment in Different Jurisdictions

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Insurance Information Institute (www.iii.org) With more than 60 insurance company members – including regional, super-regional, national and global carriers, Insurance Information Institute is an online-source for insurance information. Insurance Information Institute offers a wealth of data-driven research studies, white papers, videos, articles, infographics and other resources solely dedicated to explaining insurance and enhancing knowledge

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WHO.int (2020) This is WHO/*Europe's* portal to health statistics and to detailed monitoring and assessment tools for key areas of health policy

Risk-Benefit Considerations in Toxicology

59

Rolf Hertel, Michael Schwenk, and H. Paul A. Illing

Contents

Abstract

If an action involves risk, the outcome is not completely predictable in advance. This raises the question as to whether there are decision-making tools that could help to identify the consequences as much as possible and then to evaluate them and weigh them. Both positive consequences (benefits) and negative consequences (risks) are considered together. The evaluation depends on being able to quantify the risks and benefits using the same units, such as monetary value or length and quality of life.

Keywords

Human capital · Risk-benefit · Risk cost · Quantified risk

R. Hertel
Formerly: Federal Institute for Risk Assessment, Berlin, Germany

M. Schwenk (✉)
Tübingen, Germany

Formerly: Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

H. P. A. Illing
Formerly: Centre for Occupational and Environmental Health, University of Manchester, Manchester, UK

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https://doi.org/10.1007/978-3-030-57499-4_77

Introduction

When governments and agencies decide to take a risk, they should do so after considering formal risk-benefit and cost-benefit analyses and a utility analysis.

There are *known knowns* (those well-characterized and quantified risks and benefits), *known unknowns* (effects that can be identified but not quantified), and *unknown unknowns* (risks or benefits not known at the time of the evaluation). Risk-benefit analysis is an attempt to quantify known knowns and known unknowns, with, for the known unknowns, an attempt to quantify the uncertainties involved. It cannot address unknown unknowns, which may be risks or benefits. It is not unknown for a drug or other chemical developed for one purpose to have a much more important use discovered later – and therefore not taken into account in the original risk-benefit analysis. Also, sometimes a new toxicity is described after a drug or other chemical has been marketed that changes the risk-benefit equation. In the case of a drug, this often results in its withdrawal from the market. Thus, there is always a residuum of risk that cannot be included in a risk-benefit assessment.

Risk-Benefit Assessment

The **risk-benefit analysis** compares the identified risks with the identified benefits. A variety of methods can be used (Fig. 1). From an economic perspective, the benefit/utility of an object is based on a subjective judgment of the value of the benefit gained or the risk foregone. Both the object itself and the estimation of its value are considered. As no useful universally valid measure exists for the determination of a benefit, a benefit can only be measured indirectly. Care must be taken to

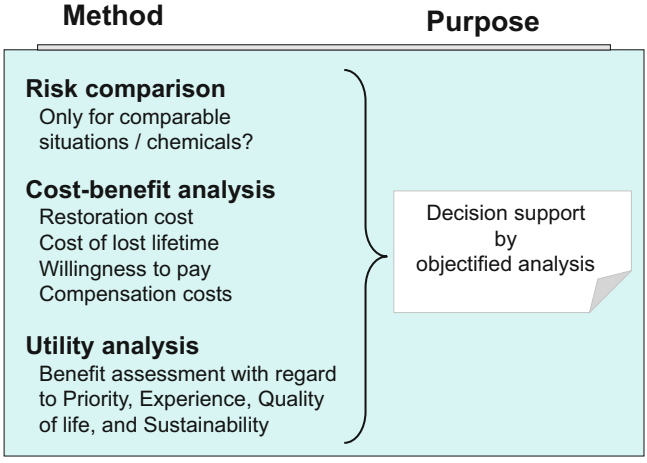

Fig. 1 Ways of carrying out a risk-benefit analysis (examples)

identify whether the property that should result in a benefit is intrinsic or whether it depends on further conditions which are not dependent on the risk decision or whether it appears just accidentally to be advantageous.

Simultaneously, both the benefits and the consequential risks must be allowed for, although these consequential risks will need to be tolerable. Household disinfectants are a good example. The aim of using the material to be evaluated is to disinfect. Disinfectants have the purpose to reduce germs. However, they can cause irritation of the skin, eye, and respiratory tract of the user if not handled appropriately. Besides the desired benefits, that is, "reducing the germs," other features, i.e., the corrosive/irritant properties of the substance, must also be taken into account. In the present example, the benefits are evident, and warning labels identifying the hazardous properties and some basic precautions inform users so that they can minimize their exposure. However, if the hazardous properties of the active ingredient are identified as an unavoidable concomitant to achieving the objective of disinfection, and serious, such a product may either be banned or some limitation as to user (e.g., "for professional use only") imposed.

From an economic perspective, there are fundamental concerns with such considerations, since the evaluation of the benefits is determined by the market and includes the question as to whether there is a demand for such a material.

Risk comparison is one method by which substitute products can transparently be evaluated. As stated in ▶ Chap. 50, "Current Role of the Risk Concept in Regulatory Toxicology," a comparison of risks is only possible if the assessable parameters of the various risks are realistically comparable, i.e., sufficiently similar in terms of their combination, chronologic order, and the distribution of advantages and disadvantages. A risk comparison can lead to a transparent identification of the critical parameters. When interpreted competently, the comparison of risks is an adequate evaluation framework in which the individual decision to take a specific risk can be made. It is important for the regulatory toxicologists that all information, including information concerning the different ways in which risks are perceived, is considered in the risk comparison. The risk comparison will only be convincing, when the underlying preconditions are clearly presented and possible shortcomings of the comparison are explained (see ▶ Chap. 58, "Risk Comparison in Toxicology").

Risk comparison may allow decision takers to communicate the decision more effectively. If the comparison shows that the risk posed by a substance or an action is less than a similar risk that has previously been accepted by society, then it is likely that the decision will be generally acceptable.

Risk comparisons are appropriate when they are based on a solid safety assessment. If however the uncertainty is high and/or variable, the risk comparison will not be convincing.

Cost-Benefit Analysis

While the risk-to-benefit analysis generally requires a more qualitative approach, a cost-benefit analysis contributes a quantitative component to the overall assessment. Here, the risks and the benefits of an action or of a substance/use are set out

quantitatively, usually in monetary terms, i.e., the risks and benefits are monetized. Consider the costs and benefits to the state of a premature death caused by a chemical exposure. The direct costs are reduced productivity and cost of treatment of any illness/infirmity. An earlier death is likely to reduce the costs of treatment of diseases of the elderly and cost of pensions. Funeral expenses will arise in both cases. Generally, possible psychological/psychiatric costs for those affected and their family are not monetized. The valuation shows here an extreme case of how profits and losses for society can be estimated. It should be noted that moral and ethical considerations may result in considerable disproportionation occurring, i.e., that the benefits gained by society may have to grossly exceed the risks taken by the individual before that risk is deemed tolerable. In some cases, moral/ethical considerations may completely override any cost-benefit analysis.

Value of a Human Life: Four different methods have been used to calculate the value of a human "life." If the costs that arise to eliminate effects of a substance or an action from which a person suffers are determined, we talk about **"regeneration cost."** This approach can be applied in connection with such accident damages that result from a risky decision. The costs can be estimated on the basis of statistical data. The monetary benefits that might result from a professional development of the person (e.g., a promotion) can however only partially – if at all – be considered.

A system which is based on years of life lost assesses the productive contribution of the individual to society. This method calculates the costs (residual working lifetime, employment rate, and national income) and the benefits which can no longer be taken advantage of (consumption, services, medical expenses). A cost calculation is based on this so-called human capital approach.

A fundamentally different method for rated human life uses neither statistical data about recovery costs nor the contribution of the individual to the productivity of the society but personal judgment. A **willingness to pay** analysis is conducted where a court awarded compensation or the amount of a life insurance serves as the base. The costs identified using this technique depend heavily on the tradition and the ethical values of the society in which the individual lives. Examples are the different levels of compensation in the USA and Europe.

Furthermore, appropriate questioning/survey techniques allow us to estimate those costs which the individual would be willing to bear in order to compensate for the consequences of an action or the application of a substance. The acceptance of such "compensation cost" depends not only on the social environment of the respondents but also on the individual's concern at the loss and the availability of alternatives, as demonstrated by the example of the marketing of so-called organically grown food.

Each method can give very different results, and the actual results obtained are country specific. In the Federal Republic of Germany, the Federal Highway Research Institute calculated the annual economic cost of road accidents. Therefore, apply in 2009 following personal accident costs: for a slightly injured person 4416 €, for a seriously injured person 110,571 €, and for a fatal outcome 996,412 €. Although the range of values is extremely broad, UK and US Governmental organizations appear to be similar. The mean values for the UK were given as 2281 million US dollars, and the USA is predictably higher, at 3472 million US dollars (Miller 2000).

Utility Analysis

Monetization is often rejected not only for ethical reasons but also because of methodological shortcomings. The utility analysis provides a quantification of values independent of money. It does not balance out cost of alternatives, but this analysis captures the benefits/utility as dimensionless value, derived from priorities, ideas, and experience of the decision-maker. The focus is on possible consequences of the decision and the probability of their arrival. If the benefits can be subdivided, the various cost-benefit values are weighted and combined to a total benefit.

It is also possible within the frame of a given plausible and/or politically legitimate objective and precondition to summarize individual cost-benefit values, determined by various decisions, and include them in the total analysis. Risk managers may choose between different courses of action, and they then have to seek widespread acceptance for the measures taken. This is best sought through a description of the decision parameters. Advantages and disadvantages should be distributed as evenly as possible among the affected individuals or interest groups. Since no pecuniary settlement is involved, even nonmonetary parameters (e.g., improved quality of life) can adequately be used for decision-making.

In utility analysis, the initial objectives and requirements in the decision-making process can continuously be questioned. If considered desirable, these objectives can be modified following appeals from stakeholders, and this should result in the greatest possible consensus (even though that may still be a very limited consensus in the case of some projects, such as new airport capacity around London!).

Cross-References

- ▶ Current Role of the Risk Concept in Regulatory Toxicology
- ▶ Ethical Issues in Science: Focus on Regulatory Toxicology
- ▶ Purpose and Methods of Risk Management in Regulatory Toxicology
- ▶ Risk Comparison in Toxicology
- ▶ Toxicological Risk Assessment

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Toxicological Risk Assessment in Different Jurisdictions

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Dietrich Henschler and Wolfgang Dekant

Contents

Abstract

The control of potential health hazards to humans due to the production, use, and disposal of chemicals is a major issue of concern. The concern arises from the increasing numbers of chemicals in production and use and the increasing numbers of chemicals demonstrated to exert toxic effects in sensitive toxicity testing systems. This situation has afforded growing legislative control of the production and application of chemicals. Control measures may limit the presence of hazardous chemicals in the environment or regulate the use of hazardous chemicals.

D. Henschler · W. Dekant (✉)
Formerly: Institute for Toxicology, University of Würzburg, Würzburg, Germany
e-mail: dekant@toxi.uni-wuerzburg.de

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Keywords

Acceptable levels of risk · Air · Chemical safety · Cosmetics · Food · Legislative control · Occupational health · Radiation · Pharmaceuticals

Introduction

The assessment of potential human *health risks* resulting from the exposure to chemicals provides the basis for appropriate regulatory and control measures (Table 1). The *health risk assessment* determines whether a chemical may cause adverse health effects, at what level, duration and frequency of exposure, and the probability that adverse health effects will occur. Risk assessment considers the available data on the toxicology of a specific chemical when judging which agents potentially pose a significant risk to the human population. Tolerable exposure levels for humans are derived from the results of animal studies by using margins of safety or defining "acceptable" incidences of adverse health effects in exposed humans.

Health risks due to contact with potentially toxic chemicals are dependent on the conditions of exposure, since not only the intrinsic toxicity of a chemical determines the magnitude of the adverse effect but also the dose. In toxicological terms, risk therefore is the product of the intrinsic toxicity of a chemical and the exposure characteristics.

Acceptable Risk, Comparison of Risks, and Establishing Acceptable Levels of Risk

In earlier phases of risk assessment, the basic belief was that few chemicals are toxic and all of these toxic chemicals are derived from synthetic processes. To achieve a zero risk, chemical exposure must be reduced below a threshold level under which it does not cause risk. However, where such a threshold cannot be demonstrated, one must assume that a finite risk may occur at any exposure level. These considerations resulted in the *zero-risk concept*. However, the more widespread testing of chemicals

Table 1 Possible measures to reduce *human exposure to hazardous chemicals*

Application of or exposure to chemical in question	Measures to reduce exposure
Industrial chemicals	Reduction or cessation of application, protective measures in the workplace, alternative chemicals with lower hazard
Pharmaceuticals	Definition of optimal dosing and dose limits, cost–benefit analysis
Alcohol, smoking, pharmaceuticals of abuse	Education
Environmental chemicals	Quantitation of exposure, strategies for avoidance, or reduction of environmental pollution

for toxicity, the increased sensitivity of analytical instruments to determine chemicals, and the developments in the science of toxicology put the basic assumption of the zero-risk concept into question.

These developments led to the recognition that zero risk was unachievable and, perhaps, unnecessary for the regulation of chemicals. This was based mainly on a few facts: (1) all chemicals, both of synthetic and natural origin, are toxic under *specific exposure conditions*; (2) most of the hazardous chemicals routinely encountered by humans are of natural rather than synthetic origin; (3) most of the exposure to hazardous synthetic chemicals cannot be avoided entirely or be eliminated from the environment without profoundly changing the way of life in many countries; and (4) in the case of cancer risk assessment, DNA damage and mutations, assumed to be of major significance in the process of carcinogenesis, occur spontaneously, albeit at a low rate.

Given these facts, the *acceptable risk concept* was developed as an alternative. The acceptable risk concept realizes that it is not possible to eliminate all potential health risks associated with chemical exposure due to the lifestyle. According to the concept of acceptable risk, safety – the reciprocal of risk – is no longer an absolute term but is redefined as a condition of certain but very low and thus acceptable risk. This conceptual change improves the ability to deal with potentially very low risks identified by the increased sensitivity of analytical instrumentation and with increasingly sensitive scientific methods to detect *potential adverse effects*. The concept of acceptable risk also permits the definition of limits for the exposure to toxic chemicals that can be considered to have a negligible impact on the incidence of adverse effects in an exposed population. Risk assessment therefore is unavoidable.

In different regulatory frameworks, while the general approaches to risk assessment are used in an identical approach – *hazard assessment*, *exposure assessment*, and *dose– response evaluation* – a number of specific factors and circumstances are influencing *risk characterization*. These are outlined in the following for some major application of chemicals. For most of the areas of applications of chemicals, the responsible authorities or scientific bodies have developed specific guidance documents which in detail define the approaches to be used and are frequently updated to include scientific progress and societal demands.

Pharmaceuticals

The marketing and application of pharmaceuticals is most highly regulated as compared to other application areas of chemicals. *Unwanted effects* play a major role in risk assessment. In contrast to other regulations concerning chemicals, which often attempt to avoid any negative health effects due to exposure, *risk–benefit considerations* are specifically integrated in the *evaluation*. Risk–benefit considerations are important since any therapeutically active chemical may have unwanted effects even at optimal *therapeutic dosage*. While unwanted effects may be mitigated by specific molecular design and optimized therapeutic schemes, they usually

cannot be completely avoided, specifically when treating life-threatening diseases. Risk assessment aims to reduce the incidence of unwanted effects to a tolerable extent. The necessary evaluation therefore includes *risk–benefit analysis*. Benefit is the *beneficial therapeutic effect* for a patient; *risk in this context is the type*, *frequency*, *and intensity of unwanted effects*. The relation between risk and benefit will then be translated in a scientifically supported relation; performing risk–benefit analysis is implied in legal regulations. However, due to the complexity of the disease processes and the potentially different responses of a disease to treatment options (from complete curability to mitigation of severe symptoms and improvement of life quality), the criteria for risk–benefit analysis in different areas of pharmaceutical treatment differ widely. For normative purposes and harmonization, science-based consensus by highly experienced expert groups and scientific societies has been developed both on a national and an international scale. *Risk assessment of pharmaceuticals* is performed at two levels, at the *level of authorization* and at the *level of supervision* of the incidence of unwanted effects when the pharmaceutical is on the market and applied to a large number of patients.

Authorization of Pharmaceuticals. Authorization of pharmaceuticals is regulated by national, European, Japanese, and US laws. Authorization by the *US Food and Drug Administration* (*US- FDA*) is often also used as the basis for authorization of pharmaceuticals by national authorities of other countries. Authorization is based on three pillars: pharmaceutical quality, measurable clinical effects, and aspects of "safety." Definition of "safety" of pharmaceuticals implies risk–benefit assessment.

The applicant, usually a pharmaceutical producer, has to submit a detailed dossier in a defined format, the *Common Technical Document* (*CTD*), to apply for authorization. All information has to be collected according to predefined protocols and to be reported in harmonized format. This common format is requested in Europe, the USA, and Japan. The CTD dossier has to contain all relevant information regarding production, research, and development of the pharmaceutical. In addition, a major focus are the results of the non-clinical, pharmacologic, and toxicological studies and all data from clinical studies investigating efficacy and frequency and intensity of unwanted effects.

Regarding toxicology, harmonization and standardization of the required toxicological testing has been developed by international harmonization between the US-FDA, the *European Medicines Agency* (*EMA*), and Japanese authorities in the *International Committee on Harmonization* (*ICH*). Most of the requested study designs developed by ICH are considered mandatory by these regulatory agencies.

Authorization within the European Union may be performed by a centralized application at the EMA or in a decentralized procedure by application to a regulatory authority of a member state. The regulatory authority evaluates the submitted dossier and may request additional information in case of uncertainties or specific issues. After authorization, effects of pharmaceuticals are further monitored to detect potential risks in larger populations. Low incidence effects with severe health impact or unwanted effects under certain conditions will only be evident after use of a pharmaceutical in large populations.

Occupational Health

Protection of worker health when handling chemicals is one of the oldest areas of regulation where a science-based risk assessment served as a basis for regulatory decisions. The major protective measures are reduction of exposure due to technical improvements of the work situation and *maximum tolerated concentrations of chemicals* in workplace air. Regarding reversible effects, *threshold limit values* (*TLVs*) are developed; for chemicals with irreversible modes of action such as carcinogens, *quantitative risk assessments* are performed. TLVs for reversible modes of action are based on thresholded dose–responses for adverse effects; concentrations below the thresholds should not result in health effects in the exposed workers. TLVs are derived from observations in occupationally exposed humans or based on animal toxicity testing using appropriate routes of exposure (usually inhalation). *Safety factors* (*SFs*) as used in risk assessment for food additives and food contaminants are not mandatory in deriving TLVs; if used, they are much smaller than those used for regulation of food additives (SFs of 5–10 as compared to 100). *Surveillance* of TLVs is performed by analytical determination of the air concentration of the respective chemical at the workplace; periodic health surveillance of exposed workers will assure that the aim of health protection is reached. TLVs are usually derived for an exposure of 8 h per day, 5 days a week, and 40-year work life, but a variety of values for shorter duration exposures or specific situations have been developed in the different regulatory frameworks. Deduction of TLVs may consider specific *individual susceptibilities* if procedures to detect such predisposing factors are available. *Sensitizing properties of a chemical* are not generally considered, but such properties will require specific labeling. Issues of costs of compliance and technical measures for compliance are not considered.

TLVs presently are also derived for chemicals with irreversible modes of action, which usually are genotoxic carcinogens. Based on the *basic concept of carcinogen risk assessment*, even very low exposures to genotoxic carcinogens may result in an increased incidence of tumors (although often in extremely low incidences); thus, the aim of complete health protection cannot be reached when exposure to a genotoxic carcinogen at the workplace may occur. Therefore, the basis for the TLVs for carcinogens is a comparison of the calculated tumor risk of an exposure (over the whole work life) with that of other occupational health risks not related to chemical exposures (such as accidents). Such a comparison requires a quantitation of the tumor risk, which is usually done by *extrapolation* of the dose–response curve from animal experimentation to render concentrations expected to result in an acceptable risk. Previously, TLVs for carcinogens were not derived since the risk assessment process has not been considered sufficiently precise. However, in Germany, since 2005, the former "Technische Richtkonzentrationen (TRK)" for genotoxic carcinogens has been formally replaced by TLVs, which need to be developed over time. Driving force for the decision was the intent to base a value for a TLV for a carcinogen on a scientific evaluation instead of relying on technical capacities, analytical surveillance, and socioeconomic issues, which were drivers for TRKs.

Risk assessments for TLVs are performed by independent scientific committees such as the "Senatskommission zur Prüfung gesundheitsschädlicher Arbeitsstoffe (MAK-Kommission)" or the EU Scientific Committee on Occupational Limits (SCOL). In analogy, TLVs in the USA are also derived by the TLV Committee. Members of the committees are recruited from independent toxicologists, occupational physicians, and analytical chemists. A public consultation period regarding the derived TLV is often integrated in the process, and all TLVs are justified in detailed written reports.

Indoor and Ambient Air

In contrast to occupational exposures which usually only occur for 8 h per day, exposure to indoor and ambient air contaminants occurs for 24 h per day and the whole population is affected. Risk assessment for ambient and indoor air contaminants often is also based on inhalation toxicity studies, but often needs to integrate higher *extrapolation factors* to cover potentially sensitive groups such as infants, the aged population, and predisposed individuals. However, besides a risk assessment-based approach using animal test data, many regulatory tolerance values regarding air contaminants are derived based on observations in large-scale epidemiology studies in humans. In addition, some values are using a precautionary approach or may be oriented regarding socioeconomic considerations and natural background of the contaminant.

Food

Food additives and *food contact materials* have become important over the past 50 years to preserve food or improve appearance and taste and shelf life. More and more contaminants present in low concentrations in food are detected by the increased food surveillance and the significantly improved analytical capabilities. Risk assessment for these types of compounds is performed on national and international levels. In the European Union, the *European Food Safety Authority* (*EFSA*) is responsible for setting tolerable concentrations of contaminants and food contact materials in different food items and for evaluation of maximum content of food additives. EFSA uses the classical risk assessment process integrating safety factors for contaminants with reversible mode of action and quantitative approaches for carcinogens, decisions on the limits are made by advisory panels of independent scientists, and detailed justification documents are published. Tolerable limits for food additives, which require authorization, are also assessed using safety factor methodology. Chemicals with genotoxic properties will not be authorized for these purposes.

Human exposures to food additives are estimated by using maximum addition levels of the additive to food items and surveillance data regarding consumption of food items containing the additive. A similar approach using concentration data for

contaminants in different food items and European consumption data are derived to define tolerable intakes for contaminants. Regarding genotoxic contaminants, the *margin- of-exposure* (*MoE*) *concept* is applied. The MoE represents the difference between the estimated human exposure to a genotoxic contaminant and a dose descriptor (usually a benchmark dose, BMD05) from animal testing. An MoE of >10,000 is considered to be of "low concern" for regulatory action or mitigation. Food contact materials are usually regulated based on *migration testing* by standardized procedures and intended uses of the food contact material with certain food items and its consumption pattern. Similar approaches as used by EFSA are used by the US-FDA and national authorities worldwide and by international organizations such as the *Joint FAO/ WHO Expert Committee on Food Additives* (*JECFA*).

Cosmetics and Consumer Products

Due to the increased awareness regarding potential risks of synthetic chemicals, risk assessment for chemical ingredients in cosmetics and consumer products is also performed. On the one hand, intentionally added ingredients are systematically assessed regarding potential health risks based on information requests by scientific advisory bodies; in addition, assessment of contaminants may be requested by regulatory authorities. Exposure assessments are performed based on the concentration of the chemical to be added to the consumer product and anticipated frequencies of use and use levels. When oral exposure is involved, migration testing determining the release of the agent from the product under predefined conditions (e.g., plasticizers from toys) is used as a major basis for exposure assessment. Regarding cosmetics, where the major exposure to ingredients is likely dermal uptake and inhalation, oral toxicity data are used as a basis for hazard assessment integrating consideration of toxicokinetics. It should be noticed that animal experiments regarding hazard assessment of cosmetic ingredients are banned in Europe after 2013 and new ingredients in cosmetics subjected to animal testing will not be permitted. Non-animal methods for hazard assessment, however, do at present not have the capacity to predict potential toxicities. A solution to this dilemma is not expected in the near future.

General Chemical Safety

The new European legislation regarding *Registration*, *Evaluation*, *Authorization of Chemicals* (*REACH*) attempts to establish "safe uses" for all chemicals available on the market and used in products. Within REACH, the producer or importer of a chemical has to file a registration dossier containing all available hazard data, anticipated exposures, and a risk assessment for the intended and foreseeable uses. The general approach therefore shifts responsibility for risk assessment from a *regulatory agency* to industry. Due to the large number of chemicals to be registered,

it is expected that only a limited number of the submissions will be checked for correctness and scientific soundness. In addition, chemicals of "very high concern" may need to be authorized for specific applications. Specific assessments of priority chemicals are made by a scientific committee (*Committee for Risk Assessment*, *RAC*) consisting of a limited number of independent scientists and mostly of representatives of national regulatory agencies. In addition, socioeconomic consequences of restrictions or bans of certain chemicals will be evaluated by a specific group. Detailed guidance regarding approaches to be used in REACH has been developed over the past decade.

Radiation Protection

Radiation protection aims at limiting the number of people exposed and the probability of exposure to be "as low as reasonably achievable" (*ALARA*); if exposed, exposure should also be as low as reasonably achievable. In the process to set exposure limits, social and economic factors are taken into consideration and risk comparisons are performed. Risk assessment for ionizing radiation for the general population is based on a comparison of the natural background radiation and its variation with that of the radiation source to be assessed. Since radiation protection assumes low-dose linearity for risk assessment with the main focus on cancer prevention, risk comparison is performed. The radiation dose expressed in Sv represents the amount of radiation energy deposited in tissue. As Sv is a large unit of measurement, the millisievert (mSv) is frequently used. The average human dose from background radiation is about 2 mSv per year. In many cases of radiation exposures, including background radiation, the radiation dose is evenly distributed throughout the body. Exposure may also be directed to a limited area of the body (radiation therapy) or single organs (e.g., radioactive iodine in the thyroid). As some organs are more sensitive to radiation, tissue weighing factors (W_T) are used to determine the equivalent risk of locally limited exposure. When the tissue weighing factor has been applied, the term "effective dose" is used. *The International Commission on Radiological Protection* (*ICRP*) recommends tissue weighing factors. The effective dose puts all ionizing radiation on an equal basis in terms of their potential to cause damage. In the EU, the upper limit for the ionizing radiation is 20 mSv/year in occupational scenarios and 1 mSv for the general population regarding technical sources.

Cross-References

- ▶ Limit Value Setting in Different Regulatory Areas of Toxicology
- ▶ Registration and Approval in Regulatory Toxicology
- ▶ The Regulatory Process in Toxicology
- ▶ Toxicological Risk Assessment

Recommended Reading

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Part VI

Risk Management and Risk Communication

International Regulation of Toxicological Test Procedures

61

Horst Spielmann and Helena Kandarova

Contents

Abstract

Differences in testing requirements in different countries, which were not based on scientific grounds, resulted in barriers to international trade and created a financial burden for the industry. Therefore, in 1981 the major industrial nations agreed to harmonize the requirements for safety testing of industrial chemicals under the leadership of the OECD (Organization for Economic Cooperation and Development). OECD Test Guidelines (TGs) are under constant review and new guidelines being adopted. Following this encouraging example, also the guidelines for safety testing of drugs and medicinal products hormones and biologicals were harmonized by the International Conference of Harmonisation (ICH) and the EU & US pharmacopoeias and safety testing of vaccines and immunologicals was harmonized by the WHO. All testing for human safety was conducted in animals and regulatory agencies wanted to accept new nonanimal tests only, if

H. Spielmann (✉)
Institute of Pharmacy, Freie Universität Berlin, Berlin, Germany
e-mail: horst.spielmann@fu-berlin.de

H. Kandarova
Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine SAS, Slovak Academy of Sciences, Bratislava, Slovakia
e-mail: helena.kandarova@savba.sk

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they allow to classify and label chemicals in the same way as the animal tests. The OECD has, therefore, decided to accept new in vitro toxicity tests, for example, for cosmetics, for regulatory purposes only after successful experimental validation. Therefore, in 1990 the European Centre for the Validation of Alternative Methods (EURL ECVAM) developed a concept for the experimental validation of toxicity test and conducted several successful validation studies with in vitro test for local toxicity on eye and skin to meet the requirements of the EU Cosmetics Directive. Since these validation studies proved successful, in 2005 the OECD published a "guidance document on the validation and international acceptance of new or updated test methods for hazard assessment." As a result, today there 16 validated OECD nonanimal toxicity test guidelines for local toxicity eye and skin toxicity and 5 for endocrine disruptors, most of them using human cells and tissues.

Keywords

Safety testing · Toxicity testing · Test guidelines · TGs · Harmonization · ICH · International Conference of Harmonisation · Good manufacturing practice · GMP · Mutual acceptance of data · MAD · OECD · Pharmacopoeia · Chemicals · Drugs · Medicinal products · Vaccines · Validation

Introduction

Toxicity testing for regulatory purposes began after the Thalidomide (Contergan®) tragedy in Germany around 1960, when unexpectedly the hypnotic drug caused severe limb malformations in newborns whose mothers had taken the drug during pregnancy. This accident initiated in the first place regulatory testing for drugs, while safety testing of other chemicals, for example, pesticides, industrial chemicals, cosmetics, and food ingredients, has become mandatory at the international level about 20 years later. Meanwhile regulatory safety testing of chemicals is mandatory in all OECD (Organization for Economic Cooperation and Development http://www.oecd.org/) member states, which are the major industrial countries except Brazil, China, and India.

Initially test requirements as well as individual tests were developed at the national level and, therefore, they differed quite significantly due to experience at the national level in industry and regulatory agencies. Consequently, international industry had to conduct different sets of safety tests to meet the legal requirements in each of these countries. Differences in test requirements created a considerable financial burden for the industry, and for patients and consumers, who in the end must pay for testing. Thus, the differences in testing requirements, which were not based on scientific grounds, resulted in barriers to international trade.

Finally, the major industrial nations agreed to harmonize the requirements for safety testing. At the OECD industry and regulatory agencies took the lead in this activity, since regulatory testing has a higher priority for them than for academic

institutions. Since it was the goal of the harmonization activity to achieve **m**utual **a**cceptance of **d**ata (MAD) by OECD all member countries, testing requirements had to be standardized. Today harmonization of test guidelines has been achieved for regulatory testing in all areas of toxicology. In addition, the criteria for development and validation of new test methods are harmonized by the OECD, since test guidelines must be updated continuously according to scientific progress and to include newly emerging endpoints.

In this chapter the concept of the international acceptance of standardized and harmonized toxicity tests (MAD concept of the OECD) is described and the criteria for developing, validating, and achieving international acceptance of new tests methods by the OECD.

International Harmonization of Guidelines for Toxicity Testing

For historical and economic reasons, standardization and harmonization of test guidelines for the major areas of toxicology, foe example, safety testing of drugs, cosmetics, industrial chemicals, pesticides/biocides, and nanomaterials, were developed separately. This is due to differences in regulatory requirements, which must cover differences in use and exposure of humans and the environment. The most important step was in 1981 the international agreement on the "OECD Guidelines for the Testing of Chemicals" (OECD TGs) http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm, which are mandatory today for testing of all chemicals except human and veterinary drugs. It is important to note that OECD TGs for safety testing of chemicals have not only been accepted for health effects (toxicity) but also for physico-chemical properties, and environmental safety including accumulation and degradation of biotic and abiotic systems. Guidelines are numbered with three-digit numbers, for example, for the health section "4" is the first number. Guidelines are under constant review, with guidelines being periodically updated, new guidelines being adopted, and guidelines being withdrawn. Previous guidelines are maintained on the website for reference purposes. To provide better understanding and faster implementation of the OECD TGs, Guidance documents (GDs) have continuously been published for specific areas of toxicity. To ensure animal welfare concerns, animal tests are only permitted where necessary.

The most important consequence of the adoption of harmonized OECD TGs by OECD member states is the concept of "**m**utual **a**cceptance of **d**ata" (MAD) that are generated by testing according to OECD TGs by regulatory agencies of all OECD member countries. Another important requirement for MAD is that testing must be conducted according to **g**ood **l**aboratory **p**ractice (GLP), which is an accepted measure to enforce quality assurance. If a toxicity test has been conducted according to an OECD TG and to GLP, the test *must* be accepted by other OECD countries for assessment purposes. This is the concept of "*tested once, accepted for assessment everywhere,*" which saves the chemicals industry the expense of duplicate testing for products marketed in more than one country.

The harmonization of TGs by the OECD has not only financial advantages for industry and consumers, but it also improves ethical standards from the animal welfare perspective, which is a major driving force in OECD member countries. Finally, from the scientific perspective harmonization of TGs is most welcome, since data for specific endpoints of toxicity must now be produced according to the same TG, which provides for a better comparison of the results of different chemicals for the same endpoint.

It has been criticized that OECD TGs are quite rigid and it is time-consuming and laborious to update them once they have been accepted, since at the OECD decisions are not made by majority vote, but all OECD member states have to agree. Although progress may be delayed by unanimous agreement, this has not really happened during the past 40 years, since the procedure for submitting new TGs and for updating existing TGs has continuously been improved.

When the EU legislation was changed in 2007 from the EU Directive 76/769/EEC on the regulation of dangerous substances and preparations to the EU Directive 1907/2006 on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), the European Chemicals Agency (ECHA), was established in Helsinki, which is only accepting data from testing according to OECD TGs (https://echa.europa.eu/guidance-documents/guidance-on-reach).

Since the concept of using standardized OECD TGs for toxicity testing has proven successful and also the MAD concept, OECD TGs are now used or safety testing of all chemical substances and products except drugs, for example, biocides, pesticides, cosmetics, as well as food and feed additives. The OECD TGs also provide the basis for the international transport of chemicals (i.e., transport regulations), although there are some variations relating to specific needs at the local level.

Considering the successful implementation of the OECD TGs into regulatory practice, in 1990 the national and international agencies that are responsible for the regulation of drug safety agreed on an international harmonization of the test guidelines for human and veterinary drugs. They were harmonized by the ICH (International Conference on Harmonisation, http://www.ich.org/), which is formed by regulatory agencies and the drug industry of the major economic regions Europe, Japan, and USA. ICH Test guidelines (TGs) are not only used for toxicity testing but also for all other areas of preclinical drug testing, for example, efficacy testing and pharmaceutical quality control. As described for OECD TGs, results of tests that were conducted according to ICH guidelines will only be accepted internationally according to MAD, if testing has been conducted according to "GLP" and produced according to "**g**ood **m**anufacturing **p**ractice (GMP)." Again, the harmonization of TGs has led to significant reduction of testing in animals, since all regulatory agencies around the world are now accepting the results of a test that was conducted according to ICH guidelines.

Table 1 summarizes the progress achieved in the international harmonization of TGs for regulatory safety testing in animals. It shows that in addition to drugs, industrial chemicals, and pesticides, TGs have also been harmonized for hormones and biologicals by the pharmacopoeias and for vaccines and immunologicals by the

Table 1 International harmonisation of test guidelines for regulatory safety testing

Industrial chemicals, cosmetics, pesticides, biocides, food and feed additives and nanomaterials:
OECD Test Guidelines for the Testing of Chemicals (http://www.oecd.org/), EU REACH Regulation, Annexes VII, VIII, IX & X (https://www.echa.europa.eu/)
Drugs and medicinal products: **ICH Test Guidelines of the International Conference for Harmonisation** (http://www.ich.org/)
H**ormones and biologicals**: **EU & US Pharmacopoeias**
Vaccines and immunologicals: **WHO Recommendations**
Medical devices testing: ISO 10993 – Biological evaluation of medical devices

WHO. So far, the harmonization of international TGs for safety and toxicity testing has been the most successful approach to reduce animal testing for regulatory purposes.

Validation and Acceptance of New Toxicity Test Methods by the OECD

Regulators will only accept new nonanimal tests, also termed "alternative tests" (e.g., in vitro or in silico tests), if the new tests allow to classify and label chemicals in the same way as the current animal tests. The OECD has, therefore, decided that in vitro toxicity tests can be accepted for regulatory purposes only after a successful experimental validation study has been conducted. To approach this problem scientifically, European and American scientists agreed in 1990 on a definition of experimental validation and the essential steps in this process. At this workshop, ***validation*** was defined as "the process by which reproducibility and relevance of a toxicity testing procedure are determined in vitro or in vivo." The essential steps of the experimental validation process were defined in the following manner: 1. test development in one or several laboratories, 2. experimental validation under blind conditions in several laboratories in a ring trial, 3. independent assessment of the results of the validation trial, and 4. regulatory acceptance.

Steps 2 and 3 are the essential part of a formal validation study conducted for regulatory purposes. The report of this workshop (Balls et al. 1990) encouraged scientists to initiate several international validation studies. Since the Draize eye test has been the most widely criticized toxicity test, a world-wide validation study on nine nonanimal alternatives to the Draize eye irritation test was co-ordinated by the EU Commission's Centre for the Validation of Alternative Methods (ECVAM, https://ec.europa.eu/jrc/en/eurl/ecvam) and the British Home Office. However, this and other extensive international validation attempts have failed (Balls et al. 1995b).

Therefore, the leading scientists recommended the inclusion of three additional elements into the validation process (Balls et al. 1995a), which had not sufficiently been identified in the ECVAM validation concept. The following three essential elements were added:

1. The definition of a biostatistical based "*prediction model*" (PM).
2. The inclusion of a *prevalidation* stage between test development and formal validation under blind conditions.
3. Well-defined *management structures*.

The PM of an in vitro test should allow the prediction of in vivo endpoints in animals or humans from the endpoints determined. The PM must be defined mathematically in the standard operation procedure (SOP) of the test that must undergo experimental validation under blind conditions with coded chemicals (Balls et al. 1995a). In order to assess the limitations of a new test before it will be evaluated in a formal validation study, the test should be standardized in a prevalidation study with a few test chemicals in a few experienced laboratories (Curren et al. 1995). This will ensure that the in vitro test method, including the PM, is robust and that the formal validation study under "blind" conditions with coded chemicals is likely to be successful.

The improved concept of experimental validation for regulatory purposes was accepted by ECVAM in 1995 and in 1996 by USA and by the OECD (OECD 1996). The improved validation concept was immediately introduced into ongoing validation studies, for example, an ECVAM/COLIPA validation study of in vitro phototoxicity tests and the ECVAM validation study of in vitro skin corrosivity tests. Since these two validations studies proved successful, in 2005 the OECD has published a "guidance document on the validation and international acceptance of new or updated test methods for hazard assessment" (OECD 2005).

Example of Successful Validation and Regulatory Acceptance of New Test Methods

Since in 1991 no standard guideline existed for the testing of photo-irritation potential for regulatory purposes, either in vivo or in vitro, the OECD, the European Commission (EC) and the European Cosmetics, Toiletry and Perfumery Association (COLIPA) established a joint program to develop and validate in vitro photoirritation tests. In the first phase of the study, in vitro phototoxicity tests established in laboratories of the cosmetics industry were evaluated and also a new assay, the 3T3 NRU PT test, which is a photo-cytotoxicity test using the mouse fibroblast cell line 3T3 and neutral red uptake (NRU) as endpoint of cytotoxicity.

In the prevalidation study conducted with 20 test chemicals, the 3T3 NRU PT in vitro phototoxicity test was the only in vitro test, in which 20 test chemicals were correctly identified as phototoxic or nonphototoxic. Quite independently, a laboratory in Japan obtained the same results in the 3T3 NRU PT with the same 20 test chemicals. In the formal validation study, which was funded by ECVAM, the 3T3 NRU PT test was validated with 30 carefully selected test chemicals in 11 laboratories in a blind trial. The results obtained in the 3T3 NRU PT in vitro test under blind conditions were reproducible, and the in vitro–in vivo correlation of the data was almost perfect (Spielmann et al. 1998a). In a subsequent blind trial on UV filter

chemicals the phototoxic potential of all test chemicals was predicted correctly in the 3T3 NRU PT (Spielmann et al. 1998b). Therefore, in 1998, the EU, having accepted the 3T3 NRU PT test as the first experimentally validated in vitro toxicity test for regulatory purposes, officially applied to the OECD for world-wide acceptance of this in vitro toxicity test. In 2000 the European Commission officially accepted and published the 3T3 NRU PT phototoxicity test in Annex V of Directive 67/548 EEC on the Classification, Packaging and Labelling of Dangerous Substances (EU Commission 1983) and in 2004 the OECD accepted the 3T3 NRU PT phototoxicity test at the world-wide level as the first in vitro toxicity test into the OECD Guidelines for the testing of chemicals (TG 432, OECD 2004).

Implementing of New Concepts for Developing Toxicity Tests During the Last Decade

Recent EU legislation, as, for example, the 7th Amendment of the EU Cosmetics Directive (EU Commission 2003), has enforced the use of nonanimal methods to replace toxicity testing in animals. Since this initiative was quite successful, for this specific field of toxicology, in vitro tests were developed, validated, and accepted by regulators, and the full ban on animal testing for cosmetic products manufactured or marketed within the EU finally came into force in 2013 (EC 2013).

The past two decades have seen unprecedented scientific and technological advances, including the birth of functional genomics, the fast-paced growth of computing power and computational biology/bioinformatics, the establishment of robotic platforms for high-throughput screening of chemicals, and the sequencing of the human genome. Together, these advances have triggered a revolution in molecular biology and have made available a wide range of new tools for studying the effects of chemicals on cells, tissues, and organisms in a rapid and cost-efficient manner.

This convergence of factors, coupled with increased recognition of the limitations of conventional in vivo tests and the need to evaluate the safety of an increasingly large number of chemical substances and mixtures, has led authorities such as the US National Research Council and other to call for shift in toxicity testing towards the elucidation of "toxicity pathways" at the cellular level – an approach commonly referred to as "Toxicity Testing in the 21st Century" (US National Research Council 2007). According to this concept, an "Adverse Outcome Pathway" (AOP) delineates the documented, plausible, and testable processes by which a chemical induces molecular perturbations and the associated biological responses which describe how the molecular perturbations cause effects at the subcellular, cellular, tissue, organ, whole animal, and population levels of observation (Ankley et al. 2010). The AOP can then be used to form categories by integrating knowledge of how chemicals interact with biological systems (i.e., the molecular initiating events) with knowledge of the biological responses. Meanwhile, the AOP concept has been accepted by the international scientific community, and the OECD launched a new program on the development of AOPs in 2013 (OECD 2013).

Table 2 In vitro toxicity tests for local eye and skin toxicity and endocrine disruption

2.1 Local eye and skin toxicity – 16 *in vitro* assays
Test No. 428: *Skin Absorption*: in vitro method
Test No. 430: *In Vitro Skin Corrosion*: Transcutaneous Electrical Resistance Test Method (TER)
Test No. 431: *In Vitro Skin Corrosion*: Reconstructed Human Epidermis (RHE) Test Method
Test No. 432: *In Vitro 3T3 NRU Phototoxicity Test*
Test No. 435: *In Vitro Membrane Barrier Test* Method for *Skin Corrosion*
Test No. 437: *Bovine Corneal Opacity and Permeability (BCOP) Test Method* for Identifying Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for *Eye Irritation or Serious Eye Damage*
Test No. 438: *Isolated Chicken Eye (ICE) Test* Method for Identifying I) Chemicals Inducing Serious Eye Damage and II) Chemicals Not Requiring Classification for *Eye Irritation or Serious Eye Damage*
Test No. 439: *In Vitro Skin Irritation*: Reconstructed Human Epidermis *(RhE) Test Method*
Test No. 442C: *In Chemico Skin Sensitization Assays* addressing the Adverse Outcome Pathway (AOP) key event on *Covalent Binding to Proteins*
Test No. 442D: *In Vitro Skin Sensitization Assays* addressing the Adverse Outcome Pathway (AOP) key event on *Keratinocyte Activation* (*ARE-Nrf2 Luciferase method*)
Test No. 442E: *In Vitro Skin Sensitization Assays* addressing the Key Events on *Activation of Dendritic Cells* of the AOP for Skin Sensitization
Test No. 460: *Fluorescein Leakage (FL) Test Method* for Identifying *Ocular Corrosives and Severe Irritants*
Test No. 492: *Reconstructed Human Cornea-like Epithelium (RhCE) Test Method* for identifying Chemicals *not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage*
Test No. 494: *Vitrigel-Eye Irritancy Test Method* for Identifying Chemicals Not Requiring Classification and Labelling for *Eye Irritation or Serious Eye Damage*
Test No. 495: *ROS (Reactive Oxygen Species) Assay for Photo-reactivity*
Test No. 496: *In vitro Macromolecular Test Method* for Identifying Chemicals Inducing *Serious Eye Damage and Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage*
2.2 Endocrine disruption – 5 *in vitro* assays
Test No. 455: *Performance-Based Test Guideline* for Stably Transfected Transactivation In Vitro Assays to Detect *Estrogen Receptor Agonists and Antagonists*
Test No. 456: *H295R Steroidogenesis Assay*
Test No. 457: *BG1Luc Estrogen Receptor Transactivation Assay* for Identifying *Estrogen Receptor Agonists and Antagonists*
Test No. 458: *Stably Transfected Human Androgen Receptor Transcriptional Activation Assay* for Detection of *Androgenic Agonist and Antagonist Activity* of Chemicals
Test No. 493: *Performance-Based Test* Guideline for Human Recombinant *Estrogen Receptor (hrER) In Vitro Assays* to Detect Chemicals with *ER Binding Affinity*

Accepted by the OECD for regulatory purposes (classification and labelling) (http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788)

Complex endpoints cannot be predicted by a single stand-alone nonanimal test as it will never be possible to reproduce a whole organism, mainly due to the lack of kinetic relationships and crosstalk among cells, tissues, and organs. It is

instead necessary to use **i**ntegrated **a**pproaches to **t**esting and **a**ssessment (IATA), where information and evidence from a battery of tests can be incorporated (OECD 2017b). Data can then be integrated by means of modeling. The IATA approach has been used successfully to replace skin sensitization testing in animals: the first three in vitro AOP-based OECD Test Guidelines (TGs) have been adopted, covering the MIE of the skin sensitization AOP (protein binding, TG 442C; OECD 2015a), as well as the intermediate key events of keratinocyte activation (TG 442D; OECD 2015b) and dendritic cell activation (TG 442E; OECD 2016). As a consequence, testing of new chemicals for local toxicity can now be conducted according to the following OECD in vitro toxicity tests methods shown in Table 2.

Table 2 summarizes OECD non-animal toxicity Test Guidelines (TGs), that have been validated according to the OECD Guidance Document 34 "*On the validation and international acceptance of new or updated test methods for hazard assessment*" (OECD 2005). Among the 16 TGs for "*local eye and skin irritation,*" there are three more complex tests for skin sensitization, which are based on the recently developed AOP and IATA concept. The 5 TGs for endocrine disruptors are addressing the interaction of test chemicals with specific hormone targets and pathways.

Outlook

In Table 2 Tests No 442C and 442D illustrate quite nicely that the new AOP concept of "toxicity pathways," which are facilitating integrated approaches to testing and assessment (IATA), can successfully be used to develop and validate new test guidelines for complex endpoints of toxicity. Therefore, the OECD is proposing that the all new tests methods should be based on the new AOP/IATA concept and it should also be considered when existing test are updated.

Cross-References

- ▶ Adverse Effects Versus Non-adverse Effects in Toxicology
- ▶ Aims and Mission of Regulatory Toxicology
- ▶ Computer-Based Prediction Models in Regulatory Toxicology
- ▶ Dose–Response Relationship and Extrapolation in Toxicology: Mechanistic and Statistical Considerations
- ▶ Institutionalized Participation in Regulatory Toxicology
- ▶ Limit Values and Guideline Values in Regulatory Toxicology
- ▶ National and International Collaboration in Regulatory Toxicology
- ▶ Risk Evaluation in Regulatory Toxicology
- ▶ Toxicity Testing In Vitro: Regulatory Aspects
- ▶ Toxicological Risk Assessment in Different Jurisdictions

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Risk Cycles in Toxicology

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H. Paul A. Illing and Michael Schwenk

Contents

Abstract

A risk cycle in toxicology typically consist of repetitive steps, consisting of hazard- and risk evaluation, risk perception, risk reduction, monitoring, and review. Risk cycles can lead to promotion of an existing risk or its reduction. Risk-reduction cycles are an effective means to stepwise minimize toxic exposures of man and the environment. Critical risk perception is an essential motor for risk-reduction efforts.

H. P. A. Illing
Formerly: Centre for Occupational and Environmental Health, University of Manchester, Manchester, UK

M. Schwenk (✉)
Tübingen, Germany

Formerly: Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

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Keywords

Risk cycle · Risk reduction · Risk management · Risk perception · Whistleblower · Pollutant

Introduction

The risk cycle involves the interplay of activities belonging to the three basic components of risk assessment, risk management, and risk communication (see "Cross-References" at the end).

Risk cycles can occur in two contrasting forms. "Risk enhancement cycles" show themselves in the form of enhanced pesticide performance (in terms of removing the pest) and enhanced levels of risk associated, e.g., with stock market speculation, willingness to partake in dangerous sports, and preparedness to escalate armed conflicts. When human behavior is involved, Adams (1995) has called this group "*Homo aleatorius*" – dice man, gambling man, or risk-taking man. They are willing to take risks, and they may be driven by mass psychological phenomena, such as a "spiral of violence."

"Risk-**reduction cycles**," however, are, in Adams' terms, undertaken by "*Homo prudens*" – those who strive to avoid "accidents." The aim is to reduce risk, based on a factual analysis and willingness to find technical solutions. This approach is rarely spectacular but is usually considered to be essential for sustainable and positive development. It occurs, for example, in the form of security measures, dispute settlement, and prevention measures. It also plays an important role in project management of companies and chemical safety.

A third variant involves no real change in risk. It is **fatalism**. Fatalists believe they have minimal control over their lives. They accept risks, are resigned to their fate and see no point in trying to change it. Thus, they are unlikely to modify the risks to which they are exposed to (Fig. 1).

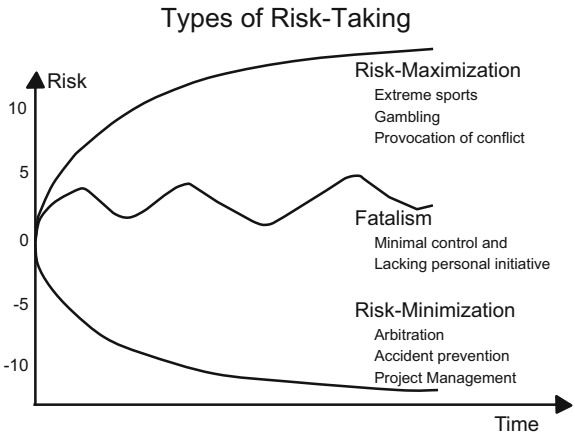

Fig. 1 Contrasting forms of risk modulation

Generally, risk taking is seen as "good" ("nothing ventured – nothing gained" or "no risk – no reward") and a necessary part of "progress." Nevertheless, the propensity to take risks, when combined with human fallibility, is often asserted to be the root causes of dangerous exposures, i.e., human error due to miscalculation, lapse of concentration, or ignorance concerning the dangers leads to inappropriate exposure. When society imposes risk management measures that reduce risk, the individual may seek to restore the balance of risk by behavior that accepts higher risks (including so-called macho behavior). Human nature often leads individuals toward *Homo aleatorius* when society as a whole wants to encourage the behavior associated with *Homo prudens*. Understanding the sociological and psychological background concerning how risks are perceived is essential if risks are to be reduced.

A Framework for Risk Evaluation

The Royal Society study group put forward a risk evaluation framework (Royal Society 1983, 1992). Essentially, this is concerned with "objective risk" – the probability that a particular adverse event occurs during a stated period of time or results from a particular challenge. It was originally developed to handle engineering risk, but it is equally applicable to health risks from chemicals. Illing and Marrs (2009) have discussed the application of this framework to the evaluation of health risks arising from exposure to chemicals.

Criteria for reaching decisions can be classified according to three "pure" criteria (UK Health and Safety Executive 2001). These are:

- An **equity-based** criterion, which starts from the premise that all individuals have unconditional rights to certain levels of protection. This leads to standards, applicable to all, held to be usually acceptable in normal life. In practice, this leads to fixing a limit to represent the maximum level of risk above which no individual can be exposed. If the risk characterization indicates that the risk is above this limit, the risk is held to be unacceptable – whatever the benefits.
- A **utility-based** criterion, which applies to the comparison between incremental benefits of measures to prevent the risk of injury or detriment (for health effects, ill-health) and the cost of the measures. The utility-based criterion compares the relevant benefits (e.g., statistical lives saved, life years extended, reduced ill health, and better quality of life) obtained by adoption of a particular risk prevention measure with the net cost of introducing it and requires that a balance be struck between the two. This balance can be deliberately skewed toward risk reduction by ensuring gross disproportion between costs and benefits.
- A **technology-based** criterion, which essentially reflects the idea that a satisfactory level of risk prevention is attained when "state of the art" control measures (technological, managerial, organizational) are employed to control risks, whatever the circumstances.

These criteria underlie the regulatory process first outlined by the Royal Society (1983). The scheme is based on:

- An upper limit of risk which should not be exceeded for any individual ("unacceptable")
- Further control, so far as is reasonably practicable, making allowances if possible for aversions to the higher levels of risk or detriment ("tolerable")
- A cutoff in the deployment of resources below some level of exposure or detriment judged to be trivial ("broadly acceptable")

This approach to risk evaluation can be applied to health effects, both to the target species and to incidentally affected species. For many health effects, the risk evaluation is concerned only with determining what constitutes a "broadly acceptable" risk and hence with the equity criterion. This is the case if any equity criterion for "safe" (the "broadly acceptable" level of risk), such as a residue level in a foodstuff, is exceeded, thus resulting in its immediate withdrawal from the market. It is also applied to the indirect risks to the environment and to humans mediated via the environment.

Risk-Reduction Cycles

The aim of risk reduction is to reduce risk levels to those regarded as "broadly acceptable" or, if this is not possible and the benefits to society are required, at least to keep risks within "tolerable" levels while seeking improvements aimed at eventually achieving the "broadly acceptable" level of risk. Measures to mitigate risks are found in many areas of human activity. Such risks can be detected early through effective project management, which includes periodical simulation of risk-reduction cycles. Here are some examples:

Product Development Risk

Different types of risks may arise during the development and use of new materials and products such as new chemicals, pharmaceuticals, and medical devices. Appropriate management can be critical for the economic success of a company:

1. The technical risk that the desired product (or the process for its manufacture) has problems and cannot be produced economically
2. The regulatory risk, that the product does not receive approval
3. The market risk that the intended sales success does not happen
4. The risks arising from litigation when a product is perceived by a consumer as having caused damaged or ill health

Reduction of product-development risks is often a consequence of product optimization.

Life Cycle Cost Risk

The life cycle cost risk is the risk of a producer or customer or their insurer. Especially for long-lived and fragile products, the long-term costs can be much higher than the purchase price. That can be considered before purchase, making a life cycle cost risk estimation. It includes not only the purchase price but also the maintenance and repair costs, disposal costs, and the possible cost for unforeseeable events ("force majeure" risks). Such a life cycle cost assessment can help to make decisions between alternative products.

Health Risks

Risk-reduction cycles have made possible the safe use of new techniques in many areas of modern life. For example, in the field of car accident prevention, the periodic improvement of occupant safety of modern cars includes introduction of crumple zones, seat belts, headrests, airbags, and antilock brakes. Comparable cycles existed in the protection of the chemical worker from harmful exposures in the workplace (occupational safety), the reduction of pollutants in consumer goods (consumer protection), and the attempts toward clean environmental media (environmental protection). Thus, in the twentieth century, the consequent reduction of uncontrolled emissions into the ambient air led to a drastic decrease of air pollution. Initially, this was through the reduction of dust emissions. Later, the emissions of sulfur dioxide, nitrogen oxides, chlorofluorocarbon propellants, lead, chlorinated hydrocarbons, dioxin, benzene, and cigarette smoke were systematically reduced. The effort is not over yet. Future tasks include the reduction of carbon dioxide, diesel particles, domestic heating emissions, garbage management, and the management of new sources of emissions.

The Risk Cycle in Toxicology

Risk-reduction cycles in the field of workers protection, consumer protection, and environmental protection typically can be divided into steps. An EU expert scientific committee has divided the cycle into four stages (EU 2000): risk evaluation, risk management options assessment, implementation of management decision, and monitoring and review (Fig. 2).

IPCS (2004) identified that risk management consists of risk or risk-benefit evaluation, emission and exposure control, and risk monitoring, with the risk options assessment being implicit in the process and risk options implementation being the decisions taken concerning emission and exposure.

In the EU scheme, phase 1, measurement organizations detect elevated levels of pollutants in the air, soil, water, or housing and suggest that this may represent a health hazard. Residents are shocked and proclaim that such a high risk is unacceptable. Health experts get involved and conclude that the risk should be avoided or

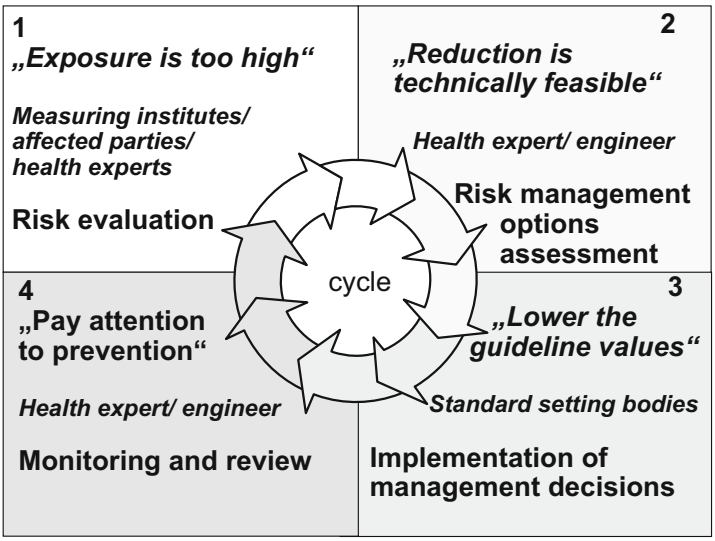

Fig. 2 The four steps of risk cycles

reduced. In EU phase 2, a range of control options are identified and an appropriate option is selected. For EU phase 2, engineers and other experts offer technical solution, leading to an emission/exposure reduction or sanitation improvement. In EU phase 3, the chosen solutions are implemented. For EU phase 4, the new level of exposure is measured and the effects evaluated. A new, and almost always tougher criterion for the exposure (normally called a standard), may be developed and the lowered guideline levels monitored. This new criterion is considered as acceptable until, some years later, new findings and insights lead again to concern, initiating a second risk-reduction cycle. Many existing limit values and guideline values were developed in the context of such cycles. When multiple cycles occur in a row, one can talk of a risk-reduction spiral.

In the environmental context, risk-reduction cycles often start in an unplanned and chaotic “scandal.” They can be promoted by “concerned” people, whistleblowers or lobbying groups, including citizens’ groups and environmental organizations. Because these groups usually demand zero risk quite aggressively (possibly combined with attempts to denigrate the experts), they initially are considered by experts (including toxicologists) with skepticism. Whenever possible, this difference between perceived risk and objective risk should be resolved as, usually, cooperation is required between all involved parties to achieve an acceptable solution.

Sometimes cycles end with an unsatisfactory result. This may be the case when the claim is too high or the solution too expensive or technically not feasible. It is also the case when obvious opportunities for improvement are ignored or when the “precautionary principle” is misapplied and a control measure introduced that is ineffective or, possibly, harmful.

Future of Risk-Reduction Cycles

Risk reduction has contributed a great deal to the steadily decreasing pollution in many parts of the world in the past decades. It has reduced the exposures of workers, consumers, and the living environment. As a beneficial side effect, the development of environmental protection technology has become an important economic benefit. The potential for further improvements is almost endless, especially when considering the global dimensions.

Sustainable development (development that meets the needs of the present without compromising the ability of future generations to meet their own needs) can and must be the way forward. But this is only possible when society considers the maintenance of a clean and healthy environment as an important goal. Experts will be required who support this idea and its implementation, both technically as well as at the regulatory level. Thus, it is likely that risk-reduction cycles will continue to play an important role in the future.

Cross-References

- ▶ Risk Comparison in Toxicology
- ▶ The Regulatory Process in Toxicology
- ▶ Toxicological Risk Assessment

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Risk Minimization in Drug Development: Regulatory Aspects

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Elke Roehrdanz and Klaus Olejniczak

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E. Roehrdanz (✉)
Federal Institute for Drugs and Medical Devices, Bonn, Germany
e-mail: elke.roehrdanz@bfarm.de

K. Olejniczak
Formerly Non-clinical Regulatory Consultant, Berlin, Germany

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Abstract

Risk minimization plays a central role in different areas of regulatory toxicology. Extremely complex and time-consuming methods are applied for risk minimization in drug development with the aim to exclude potential health risks for humans as far as possible. Therefore, the nonclinical and clinical drug development comprises a program whose results shall ensure a maximum amount of safety for each phase of clinical development (risk-benefit assessment).

Keywords

Drug development · Clinical trials · Nonclinical testing · ICH guidelines · Risk assessment

Introduction

Drug development rests on two important pillars. On the one hand, a drug has to be identified which shows efficacy in fighting or preventing a disease. On the other hand, such a drug has to be safe when administered to humans. To ensure the safe use of a drug during clinical development and later after market approval, different regulatory steps have to be taken into consideration. These steps are based on the specific drug development phase, on the patient population treated, and on the various toxicity endpoints investigated to assess potential risks for humans. This chapter tries to give an overview of the different strategies to minimize the risk during drug development.

Risk and Risk Minimization

"Risk" means that something undesirable may occur. "Probable" means that the occurrence of a risk cannot be evaluated with absolute certainty but that it will remain relative, i.e., it can be classified anything from "low" to "high," but can never be fixed at "zero" or a "100%." Therefore, nothing can ever be excluded or anticipated with certainty.

Risk minimization in the framework of drug development implies that the probability of occurrence and the extent of a possible damage caused to the health of a volunteer or patient should be kept as low as possible. Opposed to that may possibly be the entrepreneurial risk to stop the further development of a compound out of safety reasons although the compound might be generally safe and efficacious (low risk).

Risk Level

Experience and knowledge are indispensable for risk identification. If the assessment of a graded risk shows that the probability of the risk occurring is too high to be considered irrelevant (i.e., the suspicion having arisen requires clarification), then a

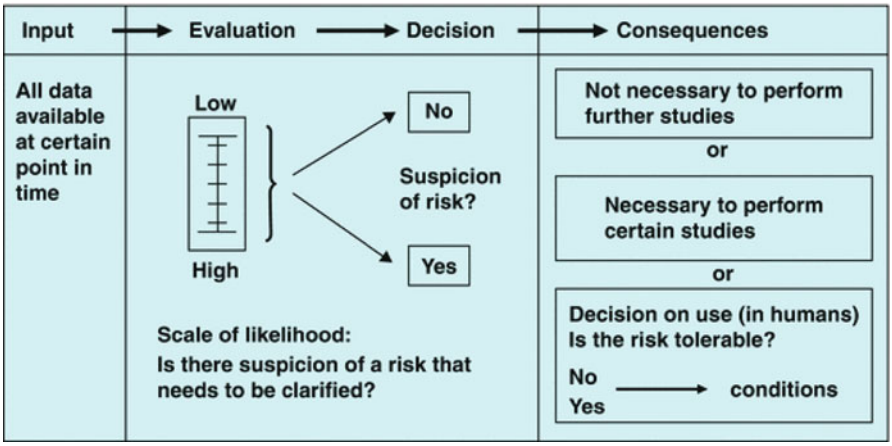

Fig. 1 Nonclinical testing strategy for risk identification

suitable nonclinical experiment must precede studies in humans. It is understandable that during medicinal product development, there will be a continuous increase in the amount of data available, which suggest a certain degree of suspicion that can only be clarified by an additional experiment or study.

The question whether there is a risk leading to such a degree of suspicion that would require scientific clarification must be answered on the basis of all available information. The assessment of the degree of risk, which ranges from "low" to "high," can only lead to one of the following decisions:

No, there is no suspicion for a risk requiring (experimental) clarification.
Yes, there is suspicion for a risk requiring (experimental) clarification.

The impact of this assessment on the requirement for particular nonclinical studies as well as on the decision about the use of the developmental compound in humans will become evident and can be categorized with the help of special flowcharts covering all typical areas of possible damage to humans. The different experimental areas are usually of interest during various phases of drug development, i.e., they will be dealt with earlier or later during drug development (Fig. 1).

Unknown Risks

Unknown risks cannot be investigated scientifically. The effects of chlorofluorocarbons (CFCs) on the stratospheric ozone layer are a well-known example. The risk from the ozone-destroying activity was discovered not until 1974, resulting in a stepwise reduction in CFC use up to complete abandonment. An analogous principle applies to medicinal uses.

Strategies for Risk Minimization in Nonclinical Development

Adaption of Trial Protocols to the Stages of Drug Development

The nonclinical development of drugs includes studies on toxicology and safety pharmacology in animals and in vitro. These studies are performed to minimize the risk for the use of a new drug substance in humans from the first orienting administration to volunteers/patients up to the broad therapeutic use in practice.

Due to average drug development times of 5–15 years, the nonclinical development program inevitably ranges over a period of several years. This program does not represent an isolated sequence of different studies but is embedded in the whole process of drug development. During drug development, a constant adaptation of both individual study plans and the whole study program to the constantly changing progress in knowledge is needed. The same applies for its execution, which happens stepwise and is coordinated with the clinical studies.

Chronological Order

Chronologically, certain nonclinical studies usually precede certain clinical trials. The required contents of nonclinical studies can be deduced from the scientific questions arising from the planned clinical trials. Generally, the characterization of the pharmacodynamic effects is followed by an investigation into toxicodynamic effects before the substance can be used in humans at all. Findings from the use in humans usually have an effect on the kind and content of further nonclinical studies and their logical and chronological position in the development process. Thus, a cycle of accumulating knowledge and its influence on the design of the remaining nonclinical program is completed. This sequence gives only an extremely rough guidance for the course of the development process for a new drug substance, regarding its contents and chronological order. However, it clearly indicates that the cycle in question can be – and usually is – repeated as development progresses.

Potential Areas of Risk for Humans

In an attempt to cover all relevant areas of potential risks, it is necessary to consider an exhaustive list of adverse reactions that are generally expected to occur and to compare them with the risks observed in the clinical trial situation. Consequently, identified and potentially meaningful risks have to be as far as possible investigated experimentally.

Typical areas of possible damage to humans are the following:

Acute toxicity
Repeated-dose toxicity

Adverse effects on reproduction (male and female fertility, embryo-fetal development, pre−/postnatal development)
Genotoxicity
Tumorigenicity
Sensitization/immune suppression and stimulation
Local or other particular adverse events

Drug development is a stepwise process where information about safety from animal as well as human studies is assessed. The aims of nonclinical studies comprise characterization of toxic effects on target organs, dose-effect relationship, relation to exposure, and potential reversibility. Such information is important regarding the evaluation of the safe starting dose in humans and to determine which parameters have to be monitored to detect possible adverse effects during the clinical trial.

Extrapolation to Humans

Before a potential medicinal product is used in humans for the first time, there are, with regard to risk assessment, only results available from nonclinical investigations and possibly some hints on potential effects in humans derived from experience with related compounds. Based on this knowledge and considering particular results from nonclinical or clinical areas under investigation, it can be stated which relation exists, e.g., between substance-related effects and amounts of bioavailable substance. It may be possible to make assumptions, whether effects were provoked by the applied substance itself or by its biotransformation products. However, at this point of time, no reliable statement can be made about the degree of similarity of the experimental models to the situation in humans.

As a result, the investigator is forced to use the potential medicinal product in a variety of testing models (various models of animal species, application forms and experimentation, and different duration of studies) in order to increase the chances of having included relevant models for the situation in humans.

Feedback of the Results from Early Clinical Trials

If this was the case can only be stated after the first studies in humans have been performed. With an increase in scientific knowledge resulting from various steps of the clinical development, certain findings obtained in the nonclinical stage will invariably lose their meaning for risk assessment.

On the other hand, through the flow of information from clinical studies, those nonclinical models can be identified that are particularly appropriate for risk assessment on the basis of their similarity to the human situation. Results from these studies together with those from early clinical trials form the basis of risk assessment, which must always be carried out before the next phase of clinical trials can be entered.

Role of Clinical Development

Clinical trials are performed in humans with the objective to show the safety and efficacy of the respective medicinal product. The first phase starts with a safe low exposure in a small number of volunteers to investigate compatibility. In the following clinical trials, exposure will generally be increased in relation to dose, duration, and/or size of the exposed patient population. Clinical trials will be extended if appropriate safety was proven in prior clinical trials plus additional safety information from nonclinical studies, which will be obtained during clinical development.

Prerequisites for Use in Humans

Prior to use in humans, it is necessary to build up a risk assessment using the most sensitive nonclinical testing model following medicinal-ethical and also legal aspects. This rule remains valid until it can be convincingly shown that the models have no or only limited biological impact on risk assessment.

The depicted approach, i.e., drawing conclusions from nonclinical test results with a view to potential results and risks for humans, and the feedback from relevant information obtained from clinical trials, turns the development and application of nonclinical testing strategies into a complex and dynamic process beyond fixed plans or checklists.

A reasonable approach implies the possibility for a critical analysis of planning, performance, interpretation, and assessment of nonclinical and clinical studies. It is acknowledged that any individual kind of investigation may be of limited relevance. One should be aware that results obtained can influence the type and extent of subsequent nonclinical and clinical studies. The design of testing strategies must, therefore, be accompanied by a high sense of responsibility reconciling the volunteers', patients', and doctors' requests for new safe medicines and the need to protect laboratory animals. Adhering to this principle will reveal, after thorough evaluation, which practical steps should be taken for each step of drug development.

Harmonization of Drug Assessment

The European Union's (EU) strive for harmonization with a view to a common market for medicinal products and the trilateral negotiations between Japan, the United States, and the EU led in October 1989 to the initiation of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). ICH is hosted by the International Federation of Pharmaceutical Manufacturers & Associations (IFPMA). The ongoing program has, among others, the following objectives:

Table 1 Nonclinical ICH guidelines: checklist

ICH code	Topic
S1A	Need for Carcinogenicity Studies of Pharmaceuticals
S1B	Testing for Carcinogenicity of Pharmaceuticals
S1C (R2)	Dose Selection for Carcinogenicity Studies of Pharmaceuticals
S2 (R1)	Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use
S3A	Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies
S3B	Pharmacokinetics: Guidance for Repeated-Dose Tissue Distribution Studies
S4	Duration of Chronic Toxicity Testing in Animals
S5 (R3)	Detection of Reproductive and Developmental Toxicity for Human Pharmaceuticals
S6 (R1)	Preclinical Evaluation of Biotechnology-Derived Pharmaceuticals
S7A	Safety Pharmacology Studies for Human Pharmaceuticals
S7B	The Nonclinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT-Interval Prolongation) by Human Pharmaceuticals
S8	Immunotoxicity Studies for Human Pharmaceuticals
S9	Nonclinical Evaluation for Anticancer Pharmaceuticals
S10	Photosafety Evaluation of Pharmaceuticals
S 11	Nonclinical Safety Testing in Support of Development of Paediatric Pharmaceuticals
M3 (R2)	Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals

To identify and eliminate the differing technical requirements in the three states/regions
To avoid repetition of all kind of tests
To accelerate drug development, thus giving patients quicker access to new medicinal products without negatively affecting quality, safety, and efficacy

In the field of nonclinical testing of drugs, 15 guidelines have been adopted since the initiation of the ICH process (Table 1).

Carcinogenicity Studies (ICH S1)

Carcinogenicity studies are generally not required to be completed prior to the conduct of clinical trials except there is a cause of concern. For pharmaceuticals, which are developed for certain life-threatening diseases, carcinogenicity studies, if applicable, may be completed after market approval.

Reproduction Toxicity Studies (ICH S5)

Reproduction toxicity studies should be conducted as is appropriate for the population that is to be exposed.

Criteria for Inclusion of Volunteers/Patients in Clinical Trials (ICH M3 (R2))

Men

Men (volunteers/patients) can be included in phase I trials and phase II clinical trials before the conduct of the male fertility study in rodents since an evaluation of the male reproductive organs is performed in the repeat-dose toxicity studies. Repeated-dose toxicity studies of at least 2 weeks are considered to be adequate for evaluation of male reproductive organs.

Women Not of Childbearing Potential

Women of not childbearing potential (i.e., permanently sterilized, postmenopausal) can be included in clinical trials without reproductive toxicity studies if the relevant repeated-dose toxicity studies (which include an evaluation of the female reproductive organs) have been conducted; again repeated-dose toxicity studies of at least 2 weeks are considered appropriate.

Women of Childbearing Potential

For women of childbearing potential, there is a high level of concern for the unintentional exposure of an embryo or fetus before information is available concerning the potential benefits versus potential risks. The recommendations on timing of reproduction toxicity studies to support the inclusion of women of childbearing potential in clinical trials are similar in the EU, United States, and Japan.

It is important to characterize and minimize the risk of unintentional exposure of the embryo or fetus when including women of childbearing potential in clinical trials. One approach to achieve this objective is to conduct reproduction toxicity studies to characterize the inherent risk of a drug. A second approach is to limit the risk by taking precautions during exposure of women of childbearing potential in clinical trials. Testing for pregnancy during the trial and subject education should be sufficient to ensure compliance with measures designed to prevent pregnancy during the period of drug exposure. To support these approaches, informed consent should be based on any known pertinent information related to reproduction toxicity. If no relevant reproductive information is available, the potential for unidentified risks to the embryo or fetus should be communicated.

In all three regions, women of childbearing potential can be included in early clinical trials without nonclinical development toxicity studies (e.g., embryo-fetal studies) in certain circumstances. One circumstance could be intensive control of pregnancy risk over a short duration (e.g., 2 weeks) clinical trials. Precautions to prevent pregnancy include pregnancy testing, use of highly effective methods of birth control, and study entry only after a confirmed menstrual period.

Generally, where appropriate preliminary reproduction toxicity data are available from two species, and where precautions to prevent pregnancy in clinical trials (see above) are used, inclusion of women of childbearing potential (up to 150) receiving investigational treatment for a relatively short duration (up to 3 months) can occur before conduct of definitive reproduction toxicity testing. This is based on the very low rate of pregnancy in controlled clinical trials of this size and duration.

In the United States, assessment of embryo-fetal development can be deferred until before phase III for women of childbearing potential using precautions to prevent pregnancy in clinical trials (see above). In the EU and Japan, other than the situations described above, definitive nonclinical developmental toxicity studies should be completed before exposure of women of childbearing potential.

In all three regions, women of childbearing potential can be included in repeated-dose phase I and II trials before conduct of the female fertility study since an evaluation of the female reproductive organs is performed in the repeated-dose studies. Nonclinical studies addressing female fertility should be completed to support inclusion of women of childbearing potential in large-scale or long duration clinical trials (e.g., phase III trials).

In all three regions, the pre−/postnatal development study should be submitted for marketing approval. All female reproductive toxicity studies and the standard battery of genotoxicity tests should be completed before inclusion, in any trial, of women of childbearing potential not using highly effective birth control or whose pregnancy status is unknown. Further details on the inclusion of women of childbearing potential in clinical trials are given in ICH M3 (R2).

Pregnant Women

Before the inclusion of pregnant women in clinical trials, all female reproductive toxicity studies and the standard battery of genotoxicity tests should be conducted. In addition, safety data from previous human exposure should be evaluated.

Clinical Trials in Pediatric Populations

When pediatric patients are included in clinical trials, safety data from previous adult human experience would usually represent the most relevant information and should generally be available before initiation of pediatric clinical trials. The appropriateness and extent of adult human data should be determined on a case-by-case basis. Extensive adult experience might not be available before pediatric exposures (e.g., for pediatric-specific indications).

Results from repeated-dose toxicity studies of appropriate duration in adult animals, the core safety pharmacology package, and the standard battery of genotoxicity tests should be available before initiation of trials in pediatric populations. Reproduction toxicity studies relevant to the age and gender of the

pediatric patient populations under study can also be important to provide information on direct toxic or developmental risks.

The conduct of any juvenile animal toxicity studies should be considered only when previous animal data and human safety data, including effects from other drugs of the pharmacological class, are judged to be insufficient to support pediatric studies.

The appropriateness of carcinogenicity testing should be addressed before long-term exposure in pediatric clinical trials. However, unless there is a significant cause for concern, carcinogenicity studies are not recommended to support the conduct of pediatric clinical trials. Further recommendations for clinical trials in the pediatric population are depicted in ICH M3 (R2) and ICH S 11.

Tolerable/Non-tolerable Risks Using the Example of Safety Pharmacology

Additionally to the characterization of the desirable pharmacodynamic effects of a drug, studies investigating secondary pharmacodynamic effects are requested. Pharmacodynamic effects relevant for safety fall into the category of "safety pharmacology." Safety pharmacology studies concerning effects of the medicinal product on vital functions like the cardiovascular, central nervous, and respiratory system should be performed prior to first administration in humans. If not covered by results from previous toxicology studies, supplemental safety pharmacology studies may be necessary for the renal/urinary system, the autonomic nerve system, the gastrointestinal system, etc. with respect to further drug development.

Example QT Interval Prolongation

A relatively recent finding is that drugs intended for a non-antiarrhythmic indication may lead to an abnormal QT interval prolongation displayed in the electrocardiogram (ECG). In this context, potential life-threatening cardiac arrhythmias belonging to the type of torsade de pointes may occur. At international level, this potentially serious adverse reaction raised a question: How can the data material collected for assessment of the arrhythmogenic potential of a drug in nonclinical studies be improved, and how can a more precise risk assessment be guaranteed?

The QT interval of the ECG is a measure of the duration of ventricular depolarization and repolarization. Repolarization of the heart ventricle is mainly influenced by the activation of the delayed rectifier K^+ current (I_k) which is composed of a rapidly (I_{Kr}) and a slowly (I_{Ks}) activating component. The rapidly activating component (I_{Kr}) is encoded by hERG (human ether-a-go-go-related gene). Substances which block the I_{Kr} prolong the action potential of the heart. Whether the medicinal product under investigation belongs to a chemical/pharmacological class with the potential to prolong the QT interval should be assessed prior to first administration in humans.

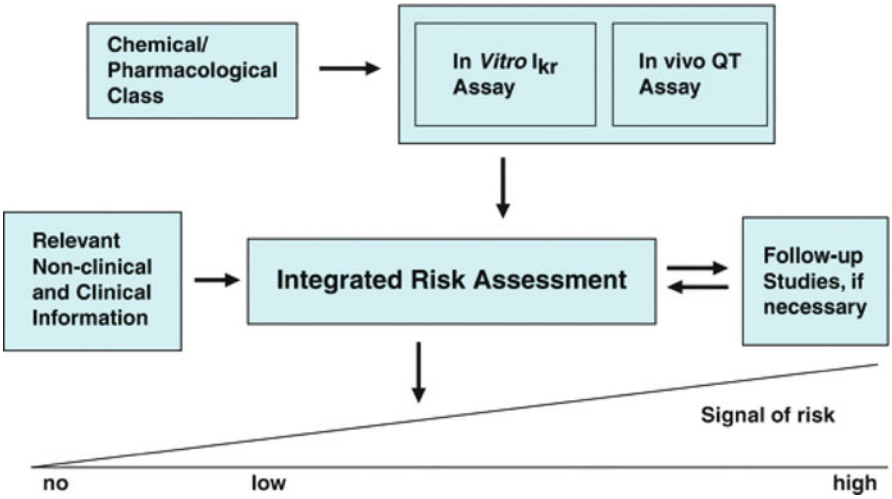

Fig. 2 Nonclinical testing strategy for assessing risk for QT interval prolongation (Refer to ICH 7B)

Nonclinical methodologies address investigations using hERG-encoded K^+ channels, action potential parameters, as well as ECG parameters taken from non-rodent species. Follow-up studies might also be necessary which, e.g., comprise investigations on different other cardiac ion channels. All data available are included in an "integrated risk assessment" to detect a potential risk for a potential to prolong the QT interval (refer to Fig. 2). The result "no risk," "low risk," or "high risk" can be crucial for further drug development. A new medicinal product with QT interval-prolonging properties has to be clearly defined concerning its therapeutic significance especially in comparison to drugs with similar or comparable indications.

Cross-References

- ▶ Ethical Issues in Science: Focus on Regulatory Toxicology
- ▶ National and International Collaboration in Regulatory Toxicology
- ▶ Registration and Approval in Regulatory Toxicology
- ▶ Toxicological Risk Assessment
- ▶ Toxicological Risk Assessment in Different Jurisdictions

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Purpose and Methods of Risk Management in Regulatory Toxicology

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Rolf Hertel, Michael Schwenk, and H. Paul A. Illing

Contents

Abstract

If the risk resulting from an event, including an event involving exposure to a defined chemical substance, is known and characterized, measures preventing the event or mitigating the damage (including ill-health) can be set up. These may include land use and emergency planning and restrictions on use, including the ultimate restriction, which is prohibition. Such measures can have major socio-economic impacts. In a democratically organized society, these measures must be acceptable to the public as a whole; thus, effective multidirectional communication between stakeholders (interested parties) is essential.

R. Hertel
Formerly: Federal Institute for Risk Assessment, Berlin, Germany

M. Schwenk (✉)
Tübingen, Germany

Formerly: Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

H. P. A. Illing
Formerly: Centre for Occupational and Environmental Health, University of Manchester, Manchester, UK

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Risk reduction · Prevention · Socioeconomic aspects · CLP · Management · Thalidomide · DDT · Smog · Prevention

Introduction

The term risk management includes those measures which must be taken by individuals or society to cope with an identified risk. The regulatory toxicologist may be asked to suggest measures that are used to control, reduce, or regulate risk. The general objective of risk management is to select appropriate tools to reduce the likelihood or size of risks and thus to prevent or minimize damage, but it may include maximizing risk to a target species with the aim of minimizing damage from, for example, a crop pest. Toxicological risk management includes standard setting, i.e., defining and setting limit values for exposure that represent the maximum acceptable or tolerable risk. Exposure standards exist within a process of risk management, which, when followed, should ensure the safe handling of the relevant substance. C*lassification and labeling* draws attention to the inherent properties without defining a risk. For a risk to exist, there must also be sufficient exposure to the substance. Exposure monitoring can then serve as management tool.

Important measures to limit the impact of unavoidable damage are appropriate land use planning, proper design of manufacturing plants, restriction on use (e.g., for certain applications or in specified processes or to specified users – often combined with the use of protective equipment) or sales outlets, and, in extreme cases, prohibitions on the marketing of certain substances. The range of these instruments shows that these measures must be decided politically. Usually there is some actual or potential benefit foregone when manufacture or use of a substance is restricted. Since the acceptability of the risk depends on attitudes to risk and in the majority of cases there is no clear, generally accepted solution, management decisions often require detailed discussion. Here, the preferences for *risk-friendly*, *risk neutral*, or *risk averse* options must be weighed against each other. Bans may ultimately lead society into a foregoing of opportunities. Therefore, such a drastic measure must be carefully justified. The choice of policy instruments for risk control is not arbitrary. The regulatory toxicologist may have to explain the basis of the acceptability of a risk in a way that is both intelligible to the public and well founded. The widest acceptance can be achieved if all stakeholders are affected similarly by the management decision. Ideally, when an individual's choice creates extra risk burdens, those burdens should be to that individual rather than to others in society or society in general.

Chemical Risk Assessment

There are two main streams of chemical risk assessment. The first is in terms of substances and their uses, and the second is in terms of risk assessment for pollution

prevention and control. The latter includes "major accident hazards," such as those seen at industrial manufacturing plants, and land use planning. Associated with the last named is the "cleanup" (remediation) for preexisting pollution.

Within the EU substances are now dealt with through the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation (Regulation EU No 1907/2006, as amended) and a variety of use-specific schemes (for medicines, veterinary products, food additives and contaminants, plant protection products, biocides, personal care products ["cosmetics"], etc.). Closely associated with this regulation is that for classification, labeling, and packaging of substances (CLP Regulation, Regulation 1272/2008). REACH and CLP replace older regulatory schemes, notably those associated with "new" and "existing" substances. REACH also subsumes the control requirements set out in former "Marketing and Use" Directives. The CLP regulation introduces the UN Globally Harmonized classifications into EU law. This classification system is intended to be utilized worldwide.

REACH requires that a chemical safety assessment is conducted for chemicals registered in quantities of 10 tons or more and chemicals identified as causing certain types of toxicity. The guidance to the REACH regulation identifies that the chemical safety report (produced as a result of the assessment) should include an assessment of any hazards that the substance may present (to human health, to physical chemical hazards [fire and explosion, etc.], and to the environment). When the substance meets classification criteria or is persistent, bioaccumulative and toxic, or very persistent and very bioaccumulative, the assessment is likely to be conducted by the regulatory authority. In all cases it includes an attempt to identify the conditions under which the risks can be controlled and therefore requires an examination of exposures and risk management procedures.

Pollution prevention and control involves both prevention and control of incidental and accidental releases of chemicals. Therefore, both land use planning and emergency planning are important. At the base of such prevention is the identification of chemicals and processes that cause harm to human health and the environment, modeling their dispersion and monitoring emitted levels of the substances to ensure that the exposures do not exceed acceptable levels. Incidental exposures are dealt with through processes such as "integrated pollution prevention and control."

In the EU, **major accident hazards** are subject to the "Seveso Directives" – Seveso 1 is Directive 82/501/EEC, Seveso 2 is Directive 96/82/EC, and Seveso 3 is Directive 2012/18/EU. This major accident hazards legislation was the result of chemical exposures following accidents at Seveso (Italy) and Flixborough (UK). The current Directive is Seveso 3 which came into effect in 2015. The legislation is concerned with both planning for the prevention of major incidents and emergency planning should a major accident occur. It should be noted that many of these major accidents are the result of fire or explosion, but others (e.g., dioxins at Seveso or methyl isocyanate at Bhopal) are due to direct health hazards of chemicals released to the atmosphere. Again, underpinning the assessment for releases of chemicals capable of causing damage to human health and to the environment is identification and characterization of the hazards (i.e., the toxicology and ecotoxicology) as well as the exposure pathways.

In certain parts of the world, "contaminated land" is an issue, i.e., land that contains substances in or under the land that are actually, or potentially, hazardous to health or the environment. This land may have been contaminated by human activities such as mining, railways, industry, chemical and oil spills, or waste disposal. Contamination can also occur naturally as a result of the geology of the area (e.g., arsenic contamination) or through agricultural use. Human exposure to contaminants can be through inhaling dust or gasses, contact with soil, or eating food grown on the land. In some cases, sites are so contaminated that they present an unacceptable risk to human health or the environment. When such contamination is identified, the land concerned requires remediation. Identification of contaminated land involves identification of the health effects of potential contaminants as well as modeling the pathways by which the contaminants are likely to be taken to the target.

Risk-Reduction Strategy

The creation of a risk-reduction strategy involves several steps, namely, risk assessment and risk or risk-benefit evaluation, identification of management options, and monitoring both of the risks and of the effectiveness of the risk management measures.

The *risk assessment*, which the risk manager receives from independent risk assessors, must be "fit for purpose." The assessment may include information on whether the statements are based on collected information or assumptions, whether extrapolation steps were included (inter- and intraspecies, route of exposure, exposure period), and, if so, what the extrapolation procedure was. Ideally, it should include an identification of any specific group within a population at higher risk. *Risk-benefit* evaluations have to take into account whether, and to what extent, benefits will be foregone as a result of the proposed risk management procedures. Occasionally, risk (or risk-benefit) evaluations will also have to take into account public opinion or, at least, opinions of affected persons ("stakeholders"), which may, in turn, be derived from controversial risk evaluations based mainly on nonscientific factors.

If the initial risk assessment results indicate to legislators that they should look further and, possibly, reduce the risk, further evaluations and adequate risk-reduction measures must be sought. A preliminary step may be to determine whether existing legislation is adequate or can be extended to cover the problem.

In REACH, the normal first step in the risk-reduction process is to perform a more specific evaluation of the risks and current management measures and, if the risks cannot be minimized to an acceptable level, a risk-benefit analysis. Technical control measures include replacement by substitution for either the chemical or the process, engineering controls – either at the design stage or by retrofitting – and use of personal protective equipment, often combined with the setting of maximum exposure standards or restricting users or outlets. Finally, in the risk-benefit analysis, socioeconomic aspects must be borne in mind. This

socioeconomic analysis requires economic and sociological expertise and includes a consideration of costs and benefits to all stakeholders of health benefits and detriments, harm to competition, and job loss. Socioeconomic analysis reduces everything to a common, usually monetary basis and is in its infancy. Possible alternative solutions can create new markets (e.g., the use of wind energy, photovoltaics).

Any decision taken must be transparent and comprehensible even though, given the complexities and inconsistencies in the available data, this may be extremely difficult. There is always a need to review and complement existing data. The adequacy and quality of the risk management measures must be checked in time in order to confirm that the required mitigation has occurred. Decisions on classification and labeling on basis of the inherent toxicity of the substance are clearly within the realm of the regulatory toxicologist. In Europe, this is done in laid down procedures in the course of chemical assessment. The decisions in this area may be subjective or a matter of judgment when qualitative factors affect the decision (e.g., extent of dermal effects) or when the relevance of animal data to humans has to be assessed (e.g., specific cancer site, molecular characteristics, and morphology).

Universally accepted decision-making patterns are emerging, even if some of these approaches are pragmatic rather than fully justified on the basis of the science.

Voluntary Agreements and Regulatory Actions

Measures concerning restrictions or even prohibitions are usually controversial. Here, two different approaches are available: *voluntary agreements* or *regulatory actions* of the legislature. It is assumed that the voluntary agreement has the advantage that it is usually considered "common sense" and shows rapid effects, while a legislative process takes a comparatively much longer time and leaves those affected without the required protection during this period. Voluntary agreements can be made, e.g., between the specific producing industry and the responsible state agency. Although historically voluntary agreements were often preferred, they have almost universally been succeeded by regulatory actions. Examples of historically important regulatory schemes are given below.

Thalidomide and Drug Legislation

In combination with other agents, thalidomide was used to treat cold, cough, anxiety, migraine, and asthma and for calming children. Very quickly it reached the largest market share in its class. At the time of its introduction, regulatory requirements were minimal, and sales in Germany and the UK increased rapidly. In 1960–1961, it became obvious that an increase in malformations in children was associated with maternal thalidomide intake, and the drug was withdrawn. The USA did not approve the drug because a theoretical possibility of

reproductive toxicity had not been evaluated experimentally. In the UK, this disaster led to an inquiry by Sir Derek Dunlop and, both in Germany and the UK, more stringent national legislation concerning the safety of medicines.

As a consequence of the thalidomide case, the UK Medicines Act of 1968 and the German Medicines Act of 1976 were enacted in order to increase drug safety. Switzerland has set up an inter-cantonal agreement in 1971. Austria followed in 1983. In recent years, the national approval systems for medicines have been subsumed into an EU-wide scheme. Generally, the legislation sets up license requirements for medicines and their producers, introduces an authorization procedure, and requires adequately conducted clinical trials. For many products, a prescription is mandatory. Recent EU legislation has introduced pharmacovigilance requirements to ensure that safety in use is properly monitored.

Smoke Control and Air Quality Legislation

The improvement of air quality with its far-reaching implications for the regulation of chemicals provides an example of successful claims regulation by new legislation.

As early as 1306, King Edward I (of England) had banned the burning of coal in furnaces in London. Almost 600 years later, the death of 1000 inhabitants of London was reported, which was caused by "smog." The smog was caused by sulfur dioxide accumulation in the air during combustion of coal. In December 1952, there was such a dense fog for 4 days ("Killer Smog") that the busses could operate only when an officer walking with a lantern showed the direction. There were 4000 extra fatalities in London hospital. It was clear from the weekly deaths registrations in the UK that the smog was the cause of death. Based on the analysis of this incident, the "Clean Air Act" was enacted in 1956 in the UK, unfortunately not soon enough to prevent further 1000 deaths in a smog period in 1955. The "Clean Air Act" set up "smoke control zones" within which emission of smoke was prohibited and controls were introduced on the fuels that could be burnt and on appliances used for burning. This Act was reinforced with the Clean Air Act of 1968 after another smog period in 1962, which cost 750 additional lives. These were consolidated into a Clean Air Act of 1993. Since 1968, there have been no similar smog episodes in London, and, as a result, chronic bronchitis and related disease clearly decreased and the number of sunny days increased. In the urban area of London, flora and fauna recovered, and there was a significant improvement in the quality of life of the citizens.

In New York, USA, there was also a smog period in 1953 which resulted in 170–260 additional casualties, and there were 405 additional cases in 1963 and 168 cases in 1966. This led to the founding of the EPA (Environmental Protection Agency, US EPA) in 1970 and the adoption of the American "Clean Air Act." The success of this law led in 1976 to the ratification of the "Toxic Substances Control Act," which authorizes the US EPA to control the use of toxic substances. In Germany, similar legislation was made possible in 1986.

DDT, Malaria Control, and Wildlife

DDT (dichlorodiphenyltrichloroethane) belongs to the class of chlorinated hydrocarbons. Its insecticidal effect was discovered in 1939 by Paul Mueller, who was honored with the Nobel Prize in 1948. Because of its low toxicity to humans – a dose of 18 g was survived – but very good efficiency against flies, lice, and mosquitoes, which transmit malaria and other diseases, its versatility, and low manufacturing and application costs, DDT quickly became the world's most important insecticide, used extensively against the vector for malaria, the anopheles mosquito. In Sri Lanka (Ceylon), some 2.8 million people suffered from malaria in 1946, i.e., before DDT spraying was used to control the vector, but only 17 cases were reported in 1963, after DDT spraying. The worldwide production and application of DDT amounted to almost 100,000 t in 1963. At this time, results were published according to which DDT is toxic to fish and causes a thinning of eggshells in birds of prey, preventing their successful reproduction. This was made public in the book *Silent Spring* by Rachael Carson in 1962. There appeared also reports that DDT generated liver cancer in mice. This together with the accumulation of the compound in human adipose tissue and breast milk resulted in attempts to ban DDT in various countries such as the USA and Germany in 1972.

In Sri Lanka, the 2.8 million cases of malaria and more than 12,500 deaths in 1946 fell to 17, and the number of deaths fell to 1 in 1963, i.e., after spraying was introduced. But 5 years after spraying ceased, i.e., in 1969, the number of deaths had climbed to 113, and the number of cases to 500,000. Selective house spraying with DDT has restored some of the control on malaria. The WHO estimates that during the 20 years of widespread use of DDT, the lives of some 100 million people living in Africa, Asia, and South America were saved.

The DDT example shows the dimensions and the dilemma of risk management decisions. Proven and assumed chronic damage of humans and animals, especially in the USA and Europe, led to a ban, which, while it may have been appropriate for the first world where malarial treatments and expensive alternative insecticides are available, caused disease and death of people in poorer, less developed countries for whom costs are critical.

It also illustrates that, generally, prevention is better than cure when examining risk management measures. Meanwhile, the situation is further complicated by the observation that DDT-resistant insects have developed.

The outbreak of the corona virus (SARS-CoV-2) pandemic in 2020 provides an example for the difficulty of reasonable risk management in a situation of unknown knowns. It was known that the virus type has a high danger potential, but its routes of infection and infectivity were initially largely unknown. The preventive actions resulted in a rapid worldwide lockdown of international traffic and new regulations, often on state or regional level, with quarantines, enforced contact restriction, and severe restriction of human freedom, sometimes in a nonproductive way. Moreover and ironically, the lockdown resulted in improvements of air quality and reduction of carbon dioxide emissions at a rate which has never been achieved by attempts of climate protection.

Preventive Measures

Preventive measures include the assessment of possible environmental and health impacts during the planning stage of projects involving potential exposure to chemicals and deciding on the acceptability or otherwise of the proposal, either for the projected facility or for the surrounding population. These may include possible considerations as to where to site a manufacturing or storage facility that is a potential major accident hazard (e.g., a chemical plant) and/or whether a (usually brownfields) site is contaminated by chemicals and requires remediation. Prevention measures (e.g., "stay indoors" while the toxic chemical disperses) may also be a part of emergency planning aimed at mitigating effects should an incident involve release of substantial amounts of a hazardous chemical. It is worth noting that farm wastes, notably those from animal housing facilities, require particular care as they can result in poisonous gasses and vapors (hydrogen sulfide, carbon monoxide, and carbon dioxide) being emitted in confined spaces (e.g., animal housing containing slurry tanks), yet agricultural buildings may be subject to less onerous planning requirements.

A **health impact assessment** (HIA) should be performed when major construction projects involving toxic agents are planned, such as town, traffic, or airport projects, waste incineration plants, wind farms, or tanks containing chlorine, ammonia, and phosgene.

Generally, the public health and environmental services are responsible for the assessment of health effects on humans. Health and environmental impact assessments are effective in the context of planning. Although they do not prevent preexisting dangers, they can also help in developing emergency procedures.

It is understandable that questions about scientific methods and administrative procedures used in toxicological risk analysis conducted by regulatory authorities are often controversial. Many of the methods used involve judgments. Topics of dispute may be the judgments concerning the quality of the data and its interpretation, judgments involved in the qualitative or quantitative risk characterization, judgments concerning the prognosis, and, especially, judgments in the risk-benefit evaluation and the attitudes to risk of the stakeholders. Many of the methods must be worked out on an international level and harmonized without increasing the administrative overhead. Actual criteria should be based on local conditions. Clearly, the people carrying out the risk assessment and management need to be properly educated and trained.

Cross-References

- ▶ Health Hazard Classification and Labelling
- ▶ Human Biomonitoring: Its Importance in Toxicological Regulation
- ▶ Importance of Exposure Level for Toxicological Risk Assessment
- ▶ Precaution Principle Versus Danger Prevention in Toxicology
- ▶ Prohibition and Restrictions in Regulatory Toxicology

▶ Risk-Benefit Considerations in Toxicology
▶ The Regulatory Process in Toxicology
▶ Toxicological Risk Assessment

Recommended Reading

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Assessment of Limit Values in Regulatory Toxicology

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Hermann H. Dieter

Contents

H. H. Dieter (✉)
Department of Toxicology of Drinking-Water, Federal Environment Agency of Germany/ Umweltbundesamt (UBA), Dessau-Roßlau, Germany

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Abstract

Limit values are legal concentration limits for chemical compounds at work, in the environment, in food, in cosmetics, in medicinal products, and so on. There are different rationales for each, but all are equally binding, both negatively and positively, for concerned persons and interest groups. These values are in constant danger of being attacked as either too stringent or too lax. For this reason, only concentration limits that have gained a maximum of societal consent by means of a transparent, politically organized process should become legally enforced. Such consent is most likely to be reached on the basis of the three-dimensional rule of environmental hygiene (REH): Avoid useless exposure, optimize functional exposure, and prevent adverse exposure.

By applying this rule, this chapter explains how to find a science-based societal consent between all interest groups when managing functional, avoidable, and natural exposure.

Keywords

Environmental hygiene · Exposure · Protection goals · Limit values · Maximal values · Precaution · Societal consent

Introduction: Types of Exposure

The criterion of "avoidability" primarily involves anthropogenic contaminants, whereas "unavoidability" concerns geogenic/biogenic exposure. With respect to tolerability (tolerance threshold T), the following regulatory differentiations must be made: on the one hand, there are the avoidable = anthropogenic exposures B and C, with their – if functional – threshold concentration $F_B > 0$ for optimal on-site technical function and $F_C \geq 0$ (remote environmental), representing the minimum concentration required to guarantee a compound's B desired on-site technical functionality (list of abbreviations for this chapter is at the end of this assay). Such exposure, provided that it is kept below a (presumed) threshold Ea of health concern about adverse effects, is either accepted at $F_B > 0$ or at $F_C = 0$, respectively, but only tolerated at levels of $T_B \geq F_B$ and $T_C \geq F_C$.

The situation is different for the virtually unavoidable = geogenic/biogenic exposure A from the use of natural resource. Here, the ratio between upper limit of expected background exposure (BG_A) and Ea is the main criterion for whether a resource A1 (with Ea $\geq BG_{A1}$) might either be usable without treatment up to a $F_{A1} \leq$ Ea or, with Ea being $<BG_{A2}$ (A2), only after reducing the compound's A2

concentration in the raw material or resource down to its technical avoidability (by elimination) and hence a tolerance threshold of $T_{A2} < Ea$.

The following conditions for *maximum* limit values LV (maximum legal concentration limits) accepted by society as a whole can therefore be derived from the rule of environmental hygiene (REH):

- *Compound class A* (*geogenic*/*biogenic*):
  - *Class A1* ($Ea \geq BG_{A1}$)
    LV = threshold of the resource's usability or $LV = F_{A1} = Ea$ (A1)
  - *Class A2* ($BG_{A2} > Ea$)
    LV = threshold of technical avoidability (elimination) or $LV = T_{A2} \leq Ea$ (A2)
- *Compound class B* (*anthropogenic*, *functional*, $F_B > 0$):
  LV = threshold of functionality or $LV = T_B \leq Ea$
- *Compound class C* (*anthropogenic*, *nonfunctional*, $F_C = 0$):
  LV = threshold of tolerability or $LV = T_C \leq \leq Ea$ (any remote T_C ought to be fixed as closely as possible to $F_C = 0$)

This classification from A to C also helps to rationalize how intensely a final product needs to be surveyed on constituents (A), residues (B), and contaminants (C). Class A compounds rarely need short-time interval surveillance. On the other hand, class B compounds need continuous surveillance at the point of on-site functional and intended addition/effect, and C compounds are preferably surveyed at more or less remote points of their unintended environmental penetration.

The concept of consensual legal limit values outlined here is not only a tool for a responsible environmental policy and surveillance. It is also an instrument that may help avoid "adverse effects" at the societal level by enabling the different interest groups to communicate with each other in a civilized and organized manner.

Criteria to Limit Exposure

Limit values (LV) are legally binding limit concentrations for chemical or other parameters in technical compartments and environmental media including food and drinking water. They have proved to regulate use and handling of chemicals and of many other noxa within all compartments of the environment and human life. They eventually quantify the *societal readiness to pay* when reducing, minimizing, or avoiding useless risks or loads on a *maximum level* and admitting functional ones on a minimal but functionally unavoidable and, hence, *minimum* technical level.

Toxicologists, health professionals, ecologists, environmental technicians, and engineers propose to legislators' options on how to substantiate necessity, nature, and numerical amount of legal limit values in the form of:

- Maximal values as derived based through science (e.g., toxicology, medicine, ecology)
- Maximum values as derived technically (functional technology and to avoid useless loads)

An ideal, politically set limit value:

- Represents the regulatory equivalent of a science-based maximum value
- Originates from a societal process of decision that is transparent and knowledge directed

When trying to scientifically find or define tolerable or acceptable maximum values for potentially harmful exposure (or to find minimum values for functional exposure), the following protection goals and corresponding options for management of their protection should be considered:

- Health of humans and their protection from illness
- Nonhuman organisms/intact ecosphere or compartments thereof
- Technical devices and equipment
- Cultural monuments and cultural traditions/customs
- Usability of natural resources
- Sensorial and expected aesthetic quality

It is clear that, within these different domains, different options are conceivable to support a maximum value for exposure towards a single compound. Any concrete numerical value (concentration) will vary in correspondence. As a consequence, in different compartments, numerically diverse limit values to trigger regulatory interventions for the same noxa are reasonable not only from the point of politics but also of science. Moreover, the nature and numerical value of a maximum value as well as the density and quality of knowledge to support it is defined by four categories of protection (see section "Categorization of Legal LVs in Terms of General Precaution, Early Warning, Control of Concern, or Control of Hazard"):

1. General precaution to prevent any possible concern
2. Early warning about possible concerns in the near future
3. Control of present concerns
4. Control of imminent hazards

If a maximum value is on the way of being established within one of these categories as a legal limit value (LV), its concrete setting as such should have brought together:

- the *scientific information* contained in that maximum value (background, warning about concern, threshold of concern, threshold of imminent hazard)with

- the corresponding *political-regulatory interpretation* and need of surveillance (precaution, early indication of possible concern, control of concern, control of hazard).

Any scientific and politically interpreted (or misinterpreted) maximum value is open to becoming judicially binding in the form of a legal limit value (LV).

Spheres of Interest When Setting Legal Limit Values

Only those maximal and minimal levels referring to societal acceptance of quality and quantity should be fixed as LVs. Such acceptance is only reached by relying on a transparent and knowledge-based societal process on how to decide which chemical or parameter must be regulated and what the subject (precaution/repair, intended/unintended, voluntary/involuntary exposure) of its regulation should be.

The central field of tension of Fig. 1 is the location of dispute and argument between:

- Political experts (e.g., members of parliament), representing the public interest for avoiding any load above socially (legally) an accepted T = *tolerance threshold*.
- Scientific experts, representing the interest of science to specify *concern about adverse-effect thresholds* = *Ea* (or corresponding risks) only if supported by correspondingly sufficient database(s). In human toxicology, as a rule, the Ea represents defined fraction of an ADI or TDI value (see section "Threshold of Concern for Adverse Effects, Ea").

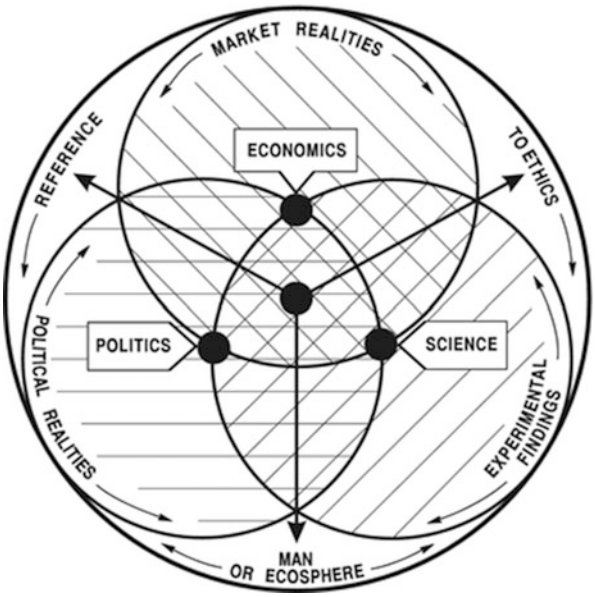

Fig. 1 Common and separated spheres of interest (overlapping areas of circles) represented by politics, the private sector, and science regarding protection of humans (health and environment) and/or ecosphere (protection of nature and its diversity). The success of consensual societal setting of LVs is possible only in the central field of tension between experimental realities (scientific considerations), market realities (beneficial and economic considerations), and political realities (consideration of democratic minorities and majorities) (Dieter et al. 1997)

- Experts from the private sector, representing the latter's interest for public acceptance of private benefit at technically consensual or optimized *functional thresholds = F.*

This complex situation was summarized by the European Environment Agency (2002): "Compartmentalised science, no matter how erudite, is an insufficient base for knowing enough to anticipate or mitigate the impacts of such systems: integrated and synthesised knowledge, which pools the wisdom from many natural and social sciences, is a necessary condition for being *Homo sapiens*."

Therefore, within the dynamics of expert panels, often only lower than purely *scientifically based and effect-related thresholds of concern* are open for societal consent or acceptance in form of a LV. Such "lower" limit values then serve (not only) to protect human health but also to reach more ambiguous technical, aesthetic, or even non-anthropocentric protection goals (Dieter 2017).

An optimal agreement in favor of an as-low-as-possible load on humans and the environment would correspond with the actual level of technical-scientific knowledge (LK) to avoid useless exposure or loads as a precaution. Less ambiguous limit values would reflect the state of technology (StT) or at least the generally accepted rules of technology (gaRT). Any LV should be set referring to three-dimensional rule of environmental hygiene (REH) as explained in the following.

Rule of Environmental Hygiene (REH)

- *Minimize useless load/exposure*. Its upper limit is *tolerated* up to T = tolerance threshold.
- *Optimize functional load/exposure*. The minimal level to guarantee intended function be F = functional threshold. F is *accepted* if set according to LK; any higher F (StT, gaRT) is *tolerated* only.
- *Prevent harmful load/ exposure*. Its upper limit is Ea = threshold of concern about adverse effects (adverse-effect threshold).

Observance of the REH not only helps to define precautionary limit values below any Ea, thus promising not only minimal necessary or even zero exposure, but also provides a perspective on a holistic social management of environmental noxa to enable "acceptable" societal consents to protect society as well from harmful social consequences.

REH-Based Criteria for Evaluating Exposure from Chemical Loads

Framework or Conditions of Social Evaluation: Prevention, Optimization/Acceptance, and Minimization/Tolerance of Exposure

The regulatory framework to apply the REH and its evaluation criteria are:

1. The (social or technical) avoidability

2. The (technical or beneficial) functionality
3. The (ecological, technical, aesthetic, or health-related) concern over adverse effects as (potentially) caused by the environmental load under question.

This framework must be delimited early on, before the factual risk assessment process.

Loads, if avoided, put aside the necessity to assess their risks. The first step before any balancing of social, beneficiary, or scientific interests when quantifying a limit value would be, at best, a decision on whether the load or exposure under question seems avoidable or not. Avoidable loads are rejected; unavoidable ones are either accepted or (temporarily) tolerated.

The decision on the tolerability of a useless load or the acceptance of a functional load should never be dramatized or trivialized by science nor be forced or prevented by economy. Each decision should follow a political rationale and be publicly defendable by referring to proven facts, functionalities, and identified opinions of majorities and minorities.

Scientists and technologists, for example, provide input to politicians with analytical data on natural background loads, on technical or other options for avoiding exposure, or on the slope and shape of a dose/response relation.

Sociologists or psychologists, on the other hand, have to determine what is the best way in a democratic society to make informed decisions that then may possibly be recognized and followed by as many (but rarely all) members of society as possible. From this societal view, that is to say, not so much the potential harmfulness of a load but much more its origin and concurrent avoidability at the point of exposure give reason for conflict, although the clashing parties often prefer to look desperately for (mostly arguable) scientific arguments to favor their specific view(s).

Holistic Concept for Evaluating Chemical Loads or Exposure in a Triangle of Precautionary Prevention, Rejection, and Acceptance/Tolerance

The point of origin or source of any anthropogenic load is always bound to a functional value or intention "on-site" in close proximity of its source. The same load, after its environmental transport to a point "off-site" or distant from the source, will have lost its functional aspect there, seeming dispensable at its place of detection. In contrast, this observation does not apply on unavoidable geogenic and/or biogenic loads, independent of any functional value or intentional context.[1]

This *first step* of evaluating or identifying a load's origin opens the option to principally reject any dispensable anthropogenic load but to principally accept or at least tolerate any geogenic/biogenic load.

However, in a *next step*, it is necessary to ask immediately for or define the possible net functional value of any load from an anthropogenic origin since, from case to case, albeit principally avoidable, such load may have been already accepted on-site at an optimized = hopefully minimized yet fully functional level.

Neither of these two decision steps is formally regulated at the societal level. Instead, the corresponding decisions on which potentially dangerous load may appear as avoidable and which one as functional as a rule are arbitrarily forced by economy and are underlined later by science, although consensual answers on which load might be "avoidable" very often could be found much easier than (often speculative) scientific answers on concerns over the adversity of a load or exposure which could or should have been avoided. Such scientific decisions are sought (but not always found) in a *third step*, called risk assessment.

This step, although often done too late (EEA 2002), is well established at the societal level. It describes and quantifies the harm (lessness) of two classes of compounds:

- *Class C of anthropogenic compounds*: These are found in "off-site" compartments, mostly environmental and distant from their primary functional use. They are therefore called *environmental contaminants*, minimized down to what are hoped are precautionary level(s) by LK or ALARA, respectively, of emission control and treatment/elimination. Examples of class C compounds are plant protection products if present *in drinking water*, HAMR if present in *surface water*, or industrial chemicals if present in *waste water*.
- *Class B of anthropogenic compounds*: These are found in "on-site" compartments, mostly technical, being close to their primary functional/intentional use. They comprise *workplace agents and additives*, *including their residues and side products*, hopefully minimized to a still fully functional level $F_B > 0$ by LK of functional optimization or (in case of side products) respecting such level. Examples of class B compounds are additives to conserve *food* and chlorine to disinfect *drinking water*, including unavoidable side products from disinfection or conservation/storage.

In both classes, load or exposure are bound to *never exceed an adverse effect threshold Ea = threshold of (health-related) concern* or to exceed an *accepted risk*, respectively. Exposure is neither allowed to damage the goal of protection nor to violate it at any possible lower level, the only exemption being a situation where a hopefully extremely high, mostly individual functional exposure is deemed to outweigh any corresponding health or functional damage or annoyance.

Close to the concrete place of their functional use and depending on the concrete binding force of exposure levels referring to LK, StT, or gaRT, respectively, *class B compounds* are tolerated by society at different minimized limits of tolerance, called *tolerance thresholds* = $T_B \geq F_B > 0$. The lowest yet still technically feasible $T_B = F_B > 0$ does conform with LK and would be *accepted* as functional by society instead of being tolerated only as would be the case if $T_B > F_B$. In any case, on this second step (see above) of the societal decision process, regulators or managers would have to allow for implementing the same or a similar functional idea only if not linked with any "unacceptable" exposure $T_B > 0$.

Class C compounds, despite being often structurally identical or closely related to those that are also found in class B, by definition are ascribed or linked only to places

and compartments where they are devoid of any functional value ($F_C = 0$). This is the reason why they never can be *accepted* there. At best, according to the binding force of levels to be defined by LK, StT, or gaRT, they may or may not even be *tolerated* at minimized limits *called tolerance thresholds* $T_C > 0$ *or even* $T_C = 0$ at/in such places and compartments.

A third class of compounds that should be clearly differentiated by management criteria from classes B and C with regard to their avoidability in a given raw material or resource are geogenic/biogenic loads or exposure. They are encompassed in the following as:

- *Class A compounds*, comprising natural constituents and their technically unavoidable transformation products.

Natural constituents, if identified or proven as potentially harmful and present at levels > Ea, are reduced by treatment down to a (technical) *tolerance threshold* $T_A <$ Ea. The numerical value of T_A, however, depends strongly on societal readiness to pay either for applying LK, StT, or simply gaRT. Examples are the elimination of inorganic arsenic from drinking water to levels far below its Ea = 10 μg/l, measures to avoid, by appropriate storage and preparation of food, the formation there of analytically detectable levels of aflatoxins, nitrosamines, or acrylamide, or the minimization of natural radioactivity, especially radon, in buildings by technical or structurally engineered measures. The *functional threshold* F_A = *threshold of usability* of such resource or space is reached at the latest if $T_A = \text{Ea} = F_A$; values of $F_A >$ Ea are then rejected together with the corresponding resource.

The definitions material to understanding the process of defining and quantifying precautionary limit values are assembled in Table 1.

Concrete Evaluation of Annoyances, Loads, and Risks Within the Triangle of Prevention, Rejection, and Tolerance/Acceptance

The numerical amount for the adverse effect threshold Ea = threshold of concern, in contrast to numerical amounts of the different categories of F and T, should, in principle, be based on strictly scientific data. This is why Ea, if either the societal tolerance of a load or exposure or the acceptance of its functional value would cease to exist, never shifts downwards nor upwards, whereas such shifting of Ea could happen by its own when reevaluating either the protection goal's sensitivity or the underlying database.

It is to be noted here that individual perception and evaluation of any "objective" risk, be this a merely supposed or an actually measured one, varies strongly with the absence or presence of a personal benefit from the same exposure and its subjective evaluation. Any *subjectively* "correct"genic Ea would vary accordingly, more so in cases where its scientific database appears vulnerable for being denunciated as not sufficient. Therefore, if a load needs to be evaluated on an insufficient or "patchy"

Table 1 Decisive maximal (max) and minimal (min) concentrations or doses of chemicals to be considered when setting precautionary limit values

Designation of maximal or minimal value	Symbol	Differentiation according to class of origin A, B, or C (see text)			
		A	B	C	Definition
Threshold (max or min) **to functionality**	**F**		F_B		Anthropogenic *on-site* concentration $F_B > 0$ of a functional chemical or its technically unavoidable residues in a final product
				F_C	Anthropogenic *remote* concentration $F_C \geq 0$ of a contaminant C or its metabolites in a final product
					(a) ***Max***: *above* which a corresponding B compound could not be allowed for use *on-site* even when applying there StT
					(b) ***Min*****:** *below* which a corresponding B compound would be excluded for use *on-site* even when applying there StT, the lowest desirable F_C being 0
Threshold (max) **to non-usability**		F_A			Geogenic concentration of a natural constituent A *above* which a resource would not be usable prior to treatment since $BG_A > Ea$
Threshold (max) **to non-tolerability**	**T**				Tolerated and/or accepted
		T_A			*Supraregional* geogenic background concentration of a natural constituent ($T_A \geq BG_A > 0$) [a]
			T_B		anthropogenic *on-site* ($T_B \geq F_B > 0$) concentration of a functional chemical or residue from it
				T_C	anthropogenic *off-site* ($T_C > F_C \geq 0$) concentration of a functional chemical or its metabolites/transformation products if presenting, e.g., at the same time as a contaminant of drinking water
Threshold (max) **for concern about adverse effects** [b]	**Ea**	No differentiation between classes of origin A, B, and C			Concentration or dose threshold above which the usability of a chemical B or of a natural resource containing a constituent A or a contaminant C would give reason for concern about adverse effects within the respective protection goal [b]

[a] BG_A represents a background concentration below T_A in any *regional* resource under specific consideration. In order to become usable, such regional resource would need elimination of a class A compound down to ≤Ea only if $BG_A > T_{A1}$ with $T_{A1} = Ea$. As long as treatment down to at least $T_{A2} = Ea$ would not seem possible or affordable, respectively, such resource remains not usable

[b] This chapter deals preferentially with potentials of adversity by threshold of effect as quantified by human toxicology. There exist, however, other thresholds whose exceedance could result in technical or aesthetic (color, odor, taste, purity) adversity or impairments/discomfort/annoyances

database, the only consensual way to proceed is to look for a level of Ea being not higher (albeit possibly lower) than a level seeming eventually quantifiable later on a then-sufficient database.

In most cases, exposure to class B compounds results in relatively high levels that are similar to exposure to class A compounds. In the absence of legal requirements to evaluate class A compounds, their toxicological database as a consequence, similar to class C, is often incomplete. Possible A risks are correspondingly often difficult to quantify but accepted more easily than B and C risks inasmuch as they appear more difficult to avoid than the latter.

Moreover, surveillance of compliance of LVs for A compounds is not necessary at the same (and high) frequency as advisable for B compounds. If the raw material or resource has been selected properly, concentrations of A compounds can be supposed to be constant, whereas concentrations of B compounds in a final product may easily be subject to technical change and failure. Finally, surveillance of C compounds should preferably be performed at the point of their environmental input and be eliminated there and not "end of pipe" having reached there a critical raw material or resource.

In any case, Ea turns out to be the only but at the same time also the maximal point of reference (health-related guide value, HRGV) to be considered when looking for a decision about which numerical amount of the different lower and much lower F_A, T_A, F_B, T_B, F_C, or T_C might appear as acceptable or tolerable to be set and surveyed in whichever frequency as a legal limit value. The numerical amount of Ea is the only merely scientific one of all these levels. It depends neither on an exposure's or loads' anthropogenic or geogenic/biogenic origin nor on whether such load or exposure may be functionally accepted or just tolerated as nonfunctional but (temporarily) unavoidable.

Definition of Precautionary Limit Values According to Origin A–C of Compounds

Class A, Geogenic/Biogenic: Natural Constituents Without (A1) or After Treatment (A2) of the Resource

Group A compounds or "constituents," according to their natural (perceived or analyzed) background concentration BG_A, the defensible effort for treatment, and the technical or health-related benefit resulting from such effort, are either eliminated from a natural resource or tolerated and even accepted, respectively.[2]

By using the respective conceptual definitions from Table 1, two relations are obtained to define precautionary limit values (LV) for class A compounds in a natural raw material or resource: one relation A1 for geogenic/biogenic compounds in resources without treatment and a second one (A2) after their treatment to eliminate class A compounds.

Definition of precautionary limit values LV_{A1} for class A1 compounds = ***constituents without treatment***:

$$0 < BG_{A1} \leq F_{A1} = T_{A1} \equiv LV_{A1} = Ea$$

The sign "≡" in this relation denominates the conceptual and numerical identity of T_{A1} from remark a) under Table 1 with the compound's legally tolerated (politically set) and mainly *natural background/health-related* limit value $LV_{A1} = Ea$ in the untreated final product. The compliance of the compound's natural concentration T_{A1} in the final product with LV_{A1} indicates social acceptance ($T_{A1} = BG_{A1}$) or at least tolerance ($T_{A1} = F_{A1}$) for utilization of natural raw material or resource, even if not treated to eliminate the critical compound.

The maximum admissible load, that is to say, the *highest possible limit value* LV_{A1} for accepting the exploitation of a natural resource without treatment, is reached if $T_{A1} = F_{A1} = LV_{A1} = Ea$.

On the other hand, by scaling the environmental quality of the LV_{A1} using the criteria offered by the REH, the decisive maximal concentration to be consensually chosen for LV_{A1} from this relation is the lowest possible concentration placed left from the sign "≡." Therefore, only if $LV_{A1} = BG_{A1}$, may the former be denominated the *lowest possible precautionary limit value* LV_{A1} to limit legally the compound's concentration in the untreated resource on its natural 50-, 90-, or any other percentile of background level (percentiles to be calculated by science and set by politics).

If weighting the relations "<" and "≤" and "=" left from any LV by 2 or 1 or zero points, respectively,[3] the weighting sum WS_{A1} takes a fraction of 3/3. This 100% *WS fraction*, if evaluating class A1 compounds according to the REH, as a rule anticipates numerical identity with Ea of a REH-compatible *precautionary* LV_{A1}.

Definition of precautionary limit values LV_{A2} for class A2 compounds = ***constituents after treatment***:

$$0 \leq F_{A2} = T_{A2} \equiv LV_{A2} \leq Ea < BG_{A2}$$

The sign "≡" in this relation denominates the conceptual and numerical identity of T_{A2} from remark a) under Table 1 with the compounds legally tolerated (politically set) and mainly *technical/treatment-related* limit value $LV_{A2} \leq Ea$ in the treated final product. The compliance of the compound's concentration T_{A2} after treatment in the final product with LV_{A2} indicates social acceptance ($T_{A2} = 0$) or at least tolerance ($T_{A2} = F_{A2}$) for the latter's utilization only if treated to eliminate the critical compound down to T_{A2} or lower.

The maximum admissible load, that is to say, the *highest possible limit value* LV_{A2} for accepting the exploitation of a treated natural resource, is $T_{A2} = F_{A2} = LV_{A2} \leq Ea$. On the other hand, by scaling the environmental quality of the LV_{A2} using the criteria offered by the REH, the decisive maximal concentration to be consensually chosen for LV_{A2} from this relation is the lowest

possible concentration placed left from the sign "≡." Therefore, only if $LV_{A2} = 0$, may the former be denominated the *lowest possible precautionary limit value* LV_{A2} to limit legally the compound's concentration in the treated resource, the technically lowest possible value of T_{A2} being a function of whether the raw material or resource was treated according to *accepted* LK or *tolerated* StT and gaRT, respectively.

The *rule of weighting* (*see with definition A1*) results in a WS_{A2} – fraction of 1/4 = 25%. This *very low fraction of* WS, if evaluating A2 compounds by applying the REH and LK for their technical elimination, anticipates only a rare numerical identity with Ea of a REH-compatible *precautionary* LV_{A2}.

Class B, Anthropogenic: Additives and Their Technically Unavoidable Residues and Side or Transformation Products

Class B compounds or "residues" deliver or delivered in their intentional and mostly technical target compartments either an *accepted* function or the presence of their transformation products, there is linked with such function in a technically unavoidable manner.

By using the respective conceptual definitions from Table 1, *the following relation to define precautionary limit values* LV_B *for class B compounds* = **residues** *in their functional technical target compartments is obtained*:

$$0 < F_B \leq T_B \equiv LV_B \leq Ea$$

The sign "≡" in this relation denominates the conceptual and numerical identity of T_B from Table 1 with the compound's legally tolerated (politically set) and mainly *technical = function-related* limit value $LV_B \leq Ea$ in the final product or technical compartment. The compliance of the compound's concentration T_B with its $LV_B \leq Ea$ in the final product or technical compartment implies social *tolerance* for the compound's functional use if $T_B > F_B$ and *acceptance* if $T_B = F_B$.

The maximum admissible load, that is to say, the *highest possible limit value* $T_B = LV_B \leq Ea$ for tolerating the functional value of a class B additive/residue/side product, is reached if $T_B = Ea$. On the other hand, by scaling the environmental quality of the LV_B using the criteria offered by the REH, the decisive maximal concentration to be consensually chosen for LV_B from this relation is the lowest possible concentration placed left of the sign "≡"; hence, $F_B = T_B \leq LV_B \leq Ea$. The F_B below which the compound's *accepted* function would no longer be realizable corresponds to LK. An $LV_B = F_B$ is the *lowest possible precautionary limit value* to limit a B compound's concentration in the final product. Values between F_B and Ea are called T_B and *tolerated* in case of applying only StT or gaRT.

Applying the *rule of weighting* (*see with definition A1*) results in a WS_B – fraction of 3/4 = 75%. This *high WS fraction*, if evaluating class B compounds by applying the REH and LK for their functional use on-site, anticipates a frequent numerical identity (between F_B and T_B) with Ea of a REH-compatible *precautionary* LV_B.

Class C, Anthropogenic: Environmental Contaminants and Their Transformation Products

Group C compounds or environmental "contaminants" and their transformation products deliver in their mostly environmental yet unintentional target compartments neither an *accepted* function nor is their presence linked there directly with such function.

By using the respective conceptual definitions from Table 1, *the following relation to define precautionary limit values* LV_C *for class C compounds = environmental* ***contaminants*** *is obtained*:

$$0 = F_C \leq T_C \equiv LV_C \leq\leq Ea$$

The sign "≡" in this relation denominates the conceptual and numerical identity of T_C from Table 1 with the compound's legally tolerated (politically set) limit value $LV_C \leq\leq Ea$. The compliance of the compound's concentration T_C with its $LV_C \leq\leq Ea$ in the environmental yet unintentional target compartment implies only social *tolerance* for the compound's presence there ($T_C > F_C = 0$) and *acceptance* only for its absence ($T_C = F_C = 0$).

The maximum admissible load, that is to say, the *highest possible limit value* $T_C = LV_C \leq\leq Ea$ for tolerating the presence of a class C compound in a "remote" environmental compartment, is reached if $T_C = Ea$. On the other hand, by scaling the environmental quality of the LV_C using the criteria offered by the REH, the decisive "off-site" maximal concentration to be consensually chosen for LV_C from this relation is the lowest possible concentration placed left of the sign "≡"; hence, $F_C = 0 = LV_C \leq\leq Ea$. If, with a $LV_C = 0$ in a remote compartment, the compound's *accepted* on-site function would no longer be realizable even by using LK, any values of T_C between F_C and Ea may be *accepted* as precautionary limit values if applying on-site LK, but would be *tolerated* only if StT or gaRT would be applied on-site.

Applying the *rule of weighting* (*see with definition A1*) results in a WS_C – fraction of 1/3 = 33%. This *low* WS fraction, if evaluating class C compounds by applying the REH and LK for their functional use on-site as B compounds, anticipates only an occasional numerical identity with Ea of an REH-compatible *precautionary* LV_C.

Setting and Evaluation of Legal Limit Values by Means of the REH

Drinking Water Limit Values from the EU as an Example

Using drinking water as an example, Table 2 demonstrates how to become informed on whether or not a legal limit value was set and quantified directly as a precautionary value by using the REH-based relations above. The legal limit values in column 4 represent a society's eventual expression of tolerance or acceptance (LV $\equiv$ T, column 4) of exposure (column 1). These LV $\equiv$ T are compared respectively and in accordance with the compounds' class of origin, with their Ea in column 5 and the author's proposal for their numerical F_B or their F_C in column 6. Two partial evaluations in columns 5a and 6a of the LV in column 4 are obtained, their absolute amount depending on whether the LV under evaluation is (much) larger, similar, or (much) smaller than the respective compound's value of Ea and of F_B or F_C. Each sign ">" ("<") counts for 2 negative (positive) weighting points, each "$\geq$" ("$\leq$") for 1 negative (positive) point, and "=" counts for ± 0. The net WS = weighting sum of all partial evaluations in column 7 eventually gives the information on whether the LV under question is a precautionary limit value (WS > 0) or whether it stands merely for nothing better than simply to defend the integrity of human health or of a technical device (WS < 0).

The method presented here to evaluate, from the aspect of environmental hygiene, legal limit values for any environmental or technical medium could easily also be applied at the beginning of any societal discourse between science, politics, and the private sector to define in advance the precautionary character of limit values to be sought by societal consent.

Categorization of Legal LVs in Terms of General Precaution, Early Warning, Control of Concern, or Control of Imminent Hazard

The expression *limit value* (*LV*) should only be used to denominate legally binding maximum values/concentrations. Legal LVs for a specific compound exhibit a strong numerical variance in correspondence with the underlying societal consensus about which goal of protection should be considered and to what extent it should be eventually protected as early as possible.

With increasing numerical ratio (hazard index) between an actual LV and an Ea value of the same compound, the setting and observation of the former departs step by step from the (1) precautionary principle over (2) early warning to enable (3) control of concern and, eventually, (4) control of imminent hazard (see section "Criteria to Limit Exposure").

This means that risk management, in case a LV would be exceeded, should be organized in accordance with the motivation underlying this LV to safeguard the goal of protection when allowing for overexposure during the LV's exceedance.

Table 2 Examples of REH-based evaluation (col. 7) of parametric values = limit values LV (col. 4) from EU "Drinking-Water" Directive 98/83/EEC. For details, see definition A, A1, B, and C of LVs in the text and remarks at end of table. Numbers for T- and F-values as proposed by author

Evaluation →	*Column 2* **highest allowable threshold = Ea** (here: health-related guide value[a]) for drinking-water)	*Column 3* **lowest possible threshold F** according to LK and class of compounds *Dimension: col. 1*	*Column 4* **tolerance threshold T** as given by EU-Directive in form of **LV≡T** *Dimension: col. 1*	**Evaluation of LV ≡ T from column 4**					
								Column 7	
↓ **Compound** (class of origin)				*Column 5* **comparison** of Ea (col. 2) with LV (col. 4)	*Column 5a* **+bonus/-malus** for LV in col. 4 by comparison with T in col. 5	*Column 6a* **+bonus/-malus** for LV in col. 4 by comparison with F in col. 6	*Column 6* **comparison** of F (col. 3) with LV (col.4)	**Bonus *plus* Malus** from summing col.s 5a and 6a	**corresponding qualitative evaluation of the LV in col. 4**
Arsenic (A2)	10 µg/l	F_{A2}[b]=**Ea**=10	T ≡ **LV** = 10[c]	**Ea** ≡ T →	**±0**	**±0**	← F = T	**±0**	**LV✓?**
Lead (B)	10 µg/l	$F_B = 40$	T ≡ **LV** = 40[d]	**Ea** << T →	**−2**	**±0**	← F = T	**−2**	**LV ↓↓!**[d]
Bromate (B)	0.3 µg/l	$F_B = 10$	T ≡ **LV** = 10	**Ea** << T →	**−2**	**±0**	← F = T	**−2**	**LV ↓↓!**
Cadmium (A1)	3 µg/l	F_{A1}[b] = **Ea** = 3	T ≡ **LV** = 5[a]	**Ea** < T →	**−1**	**−1**	← F < T	**−2**	**LV ↓↓!**[a]
Cyanide (A1)	50 µg/l	$F_{A1} = BG_{Al}$[c] < **Ea**	T ≡ **LV** = 50	**Ea** = T →	**±0**	**−1**	← F < T	**−1**	**LV ↓!**
1.2-Dichloro-ethane (C)	3 µg/l	$F_C = 0.00$	T ≡ **LV** = 3	**Ea** = T →	**±0**	**−1**	← F < T	**−1**	**LV ↓!**
Fluoride (A1)	1.5 mg/l	F_{A1}[b] = **Ea** = 1.5	T ≡ **LV** = **Ea**	**Ea** = T →	**±0**	**±0**	← F < T	**±0**	**LV✓?**
Copper (B)	2 mg/l	$F_B = 2$	T ≡ **LV** = 2	**Ea** = T →	**±0**	**±0**	← F = T	**±0**	**LV✓?**
Chlorinated solvents (C)	20←→100 µg/l	$F_C = 0.00$	T ≡ **LV** = 10.0	**Ea** > T →	**+1**	**−1**	← F < T	**+0**	**LV✓?**

Manganese (Mn^{+2}) (A2)	1.0 mg/l (E) 0.2 mg/l (**Ea**)	F_{A2}[b]=**Ea**[f]=0.05	T ≡ **LV** = 0.05[c]	**Ea** = T →	**±0**	**±0**	← F = T	**±0**	**LV✓?**
Nitrate (C)	50 mg/l	F_C=20	T ≡ **LV** = 50	**Ea** = T →	**±0**	**−1**	← F < T	**−1**	**LV ↓!**[g]
PAH (A1; B)[h]	≥ 0.1 µg/l	F_{A1} = 0.1[e]≤**Ea**	T ≡ **LV** = 0.10	**Ea** ≥ T →	**+0.5**	**±0**	← F = T	**+0.5**	**LV✓!**
Pesticides (C)	1←→1,000 µg/l	F_C=0.00	T≡**LV**=0.10	**Ea**>>T →	**+2**	**−1**	← F < T	**+1**	**LV✓!**
THMs (B)	60–200 µg/l	F_B =10[i]	T ≡ **LV** = 10[i]	**Ea** > T →	**+1**	**±0**	← F = T	**+1**	**LV✓!**

✓! **means**: **Bonus** + **Malus** > **0**. The actual **LV** ≡ T for these compounds may be evaluated without any restriction as a precautionary maximal value far below any Ea

! **means**: **Bonus** + **Malus** < **0**. The actual **LV** ≡ T for these compounds is not a precautionary maximal value. It is even too high (↓↓!) if interpreted as adverse effect-related LV (its lowering seems necessary)

✓? **means**: **Bonus** + **Malus** = **0**. **LV** ≡ T for these compounds may yet be interpreted as a precautionary maximal value but also to be adverse effect related. Its compliance therefore needs regular short-time interval surveillance

[a]From WHO (2017), **LV** ≡ T for cadmium should be lowered for health reasons to Ea = 3 µg/l (health-related guide value of WHO 2017)

[b]Threshold to usability of a raw water if not treated accordingly to eliminate potentially harmful geogenic/biogenic constituents.

[c]After treatment

[d]EU-LV since November 1998 (with moratorium): 10 µg/l, to be met only after exchange of lead pipes by pipes made from better adapted materials

[e]Upper limit of geogenic load (cyanide) or of geogenic background (PAH) plus unavoidable load from using old coal tar-coated pipes (numbers estimated by author)

[f]Threshold to *technical* adversity

[g]The LV for nitrate is the only one (!) for an environmental contaminant C in drinking water with an exclusively health-related rationale

[h]Sum of four polycyclic aromatic hydrocarbons (PAH, without benzo[a]pyrene) originating from old coal tar-coated pipes and/or geogenic/biogenic background

[i]To be complied with at outlet of waterworks

General Precautionary Maximal Values, PV_g

General precautionary maximal values are the numerically lowest of all possible LVs. They help to avoid from the start loads and annoyances, not just when looking at specific protection aims but rather in general for present and future generations. For compounds of classes A1 and C, such PVs ideally ask for not exceeding a compound's natural (regional?) background concentration. For class C compounds, this condition mostly would mean a (analytical) level "zero," realizable at best by LK or ALARA, whereas precautionary LVs for class B or A2 compounds, as a rule, are to be found between classes C and A1 since they are situated close to or are identical with the lowest level of technical feasibility (of treatment or function, respectively).

The best rationale of a broadly accepted PV would be an equal balance between scientific quantification, technical/functional benefit, and political acceptance/tolerance of any corresponding risk or load. Their scientific part, as a rule, is confined to quantifying natural background concentrations or high-quality analytical criteria to allow for reliable detection or definition, now and in future, of any deviation of load/exposure from a legally accepted (e.g., "background") PV_g.

Only a few of the LVs in Table 2 for A1 and C compounds or A2 and B compounds, respectively, are close to their ideal (optimal precautionary) maximal contaminant level as described by this condition.

Sustainable repair of a precautionary LV's exceedance to achieve legal recompliance normally is not adequately feasible by means of short-time measures to protect persons or technical devices from immediate hazards or risks. Instead, such sustainable repair ought to be considered on a medium to long-term time scale by using the scope of sustainable action, as it should be part of any precautionary LV concept. For the time of repair, so-called health-related "maximal action values" may be functional to rationalize and avoid risks from possible but temporarily limited exposure $>$ LV.

Within the framework of this discussion, general PVs are conceptually and numerically identical, respectively, with F_C, F_B, or BG_{A1}.

Warning Values, WV

The next higher and, from a scientific point of view, sounder category of maximum values is the category of *warning values*, *WVs*. Their exceedance should indicate as early as possible, on the basis of scientific data, that the normal state of a system or organism could be undergoing a switch to instability or non-normality. The database of a WV is more informative than that of a general precautionary value; therefore, WVs are also called specific (health related, technical, sensorial/aesthetic) precautionary values. As such, they do not need to be "LK," but should at the same time never be higher than any science-based threshold of concern as possibly derived later on a sufficient database to replace such warning value.

Within the context of this discussion, WVs are conceptually and numerically identical, respectively, with any T_C, T_B, or T_{A1}. For the special case of human toxicology, such WVs have been denominated in Germany as *GOW = Gesundheitlicher Orientierungswert* (in English, *HRIV = health-related indication value*). The scientific basis of this pragmatic regulatory approach was outlined by Dieter (2014).

Threshold of Concern for Adverse Effects, Ea

The exceedance of a scientifically based *threshold of concern for adverse effects*, Ea, would not just warn about a possible health concern for adverse effects in the future but should also directly trigger such concern. Toxicologists derive an Ea in a way that the selected protection goal is unlikely to be harmed as long as the measured load/exposure is in compliance with the same compounds' Ea.

In human toxicology, an Ea, as a rule, is derived from an ADI or TDI value by allocating a certain percentage from the latter to the amount in kilograms or liters of daily personal consumption (e.g., 2 l of drinking water or 1 kg of food). In the case of drinking water, such aliquot concentration normally represents 10% of an ADI or TDI and is usually called a compound's health-related guide value, HRGV, for drinking water (in German LW = *gesundheitlicher Leitwert*).

Hazard-Linked Action Values, AV

Scientifically based maximum values whose exceedance in a standard scenario would trigger with sufficient probability an imminent hazard from toxic exposure are called *hazard-linked action values*, AV. They are higher by a compound-specific "interpolation factor" (IF) than the scientific Ea of the same compound.

In Germany, as a rule, AVs are calculated as being threefold to tenfold higher than a corresponding Ea. On a quasi logarithmic scale, the interpolation into the margin of safety, which is the space of adverse effect extrapolation from experimental conditions on humans, places the AV halfway between the selected PoD = point of departure of extrapolation and the protection goal's Ea (Dieter and Konietzka 1995; Konietzka and Dieter 1998; Dieter 2011).

Summary: A Short Directory to Quantify and Survey Precautionary LVs

The REH helps to organize the following steps to fix socially consensual (tolerated or accepted) limit values and criteria for their surveillance:

- A: Geogenic/biogenic constituents (hardly avoidable):
  - A1: If no single threshold of concern Ea is exceeded in the raw material or resource, respectively, treatment to eliminate class A compounds would neither be necessary nor indicated. Any LV_{A1} to be chosen accordingly would appear as tolerable, if representing rather an "upper" percentile, or as acceptable, if representing rather a "lower" percentile of regional BG_{A1} levels.
    As a rule, any $LV_{A1} \equiv BG_{A1}$ would need only a longtime interval surveillance to safeguard compliance.
  - A2: If one or more threshold(s) of concern Ea are exceeded in the raw material or resource, respectively, treatment(s) to eliminate class A compounds down to a technically tolerable (gaRT) or acceptable (StT or LK) level of

$LV_{A2} \equiv T_{A2} \leq Ea$ is indicated only if a resource to need no such treatment would not be readily (e.g., regionally) available.

As a rule, any $LV_{A2} \equiv T_{A2}$ would need a short-time interval surveillance in the treated resource to safeguard compliance.

- B: Anthropogenic additives with intended function in specific compartments with their residues and unavoidable transformation products:
  B compounds ought to exert their intended function as far as possible below their on-site threshold of concern Ea. Any LV_B to be chosen accordingly would appear as being tolerable at a level of $LV_B \equiv T_B \leq Ea$ (gaRT) or as acceptable as a precautionary maximal value at a level of $LV_B \equiv T_B = F_B < Ea$ (StT or LK). The societal readiness to *accept* any levels $T_B > F_B$ below Ea increases with optimization of functional efficiency and increasing readiness to pay for them.

  As a rule, any $LV_B \equiv T_B$ would need a short-time interval surveillance in the finished product to safeguard compliance.
- C: Anthropogenic environmental contaminants and degradation products in remote places and compartments where they are devoid of intended function and potentially harmful:
  C compounds may dissipate unintentionally from the on-site use of B compounds onto remote places or compartments of the environment (resources or raw materials). If there is no single or sum threshold of concern Ea exceeded, their treatment to eliminate class C compounds would neither be necessary nor indicated. An LV_C to be chosen accordingly would appear as tolerable at a level of $LV_C \equiv T_C < Ea$ (gaRT) and as acceptable as a precautionary maximal value only at the level $0 = F_C = T_C \equiv LV_C$ (StT or LK). The societal readiness to *tolerate* any remote levels $T_C > F_C$ below Ea increases with optimization of functional compartmentalization of the use on-site of the respective class B compounds and increasing readiness to pay for.

  As a rule, any $LV_C \equiv T_C$ would ask for a longtime interval surveillance to safeguard compliance in any remote contaminated raw material or resource if this is done in alliance with the respective class B compound's short-time interval on-site surveillance of emission and degradation/dissipation.

Conclusions

The three-dimensional *rule of environmental hygiene* (REH) as outlined here serves to ascertain in each individual case whether a limit value is, in fact, necessary to avert contamination and, if so, which component of the overall rationale determines or should determine where and at which level at which it should be fixed. This assessment has to take place by way of a rational public discourse befitting democratic forms of government, with the participation of all societal players (cf. AdW 1992; EEA 2002; Dieter 2017). Only such procedure opens the way to avoid exposure inequities, differences in acceptance, and uncertainty as to the conditions governing economic activities within society. Violators of limit values coming from such procedure rightfully face society's sanctions.

Limit values also serve to establish an overall social compatibility of exposure whose implementation is useful to one party, of no particular interest to another, and possibly detrimental to a third. This compatibility should best be cleared before questions of whether an adverse effect threshold is reached or exceeded can or must be answered by science in a reliable manner.

The three-dimensional concept to fix limit values outlined here is an instrument that may also be helpful in limiting "adverse effects" at the societal level by enabling different interest groups to communicate with each other in a civilized, organized, tolerant, and, hence, acceptable manner.

Those who permanently or temporarily withdraw from such rational, civilized discourse in order to push through, out of self-interest, highly specific protection goals aside from general acceptability deliberately act in an anti-democratic or a politically short-sighted manner and are in danger of losing their credibility.

On the other hand, it is only in rare cases that the temporary exceedance of a limit value directly results in an immediate hazard. Information on such "dangerous" values can be obtained from toxicologists and ecologists, who should have no reservations about divulging it. The overwhelming majority of limit values, however, have been found and fixed on the basis of criteria relating to the functional value of useful exposure and the avoidability of unnecessary exposure – at levels usually far below those based on the criterion of "imminent hazard."

Thus, the often-heard slogan of "poisoning sanctioned by limit values" is quite out of place. All the more care must be taken to ensure that limit values are complied with and that remedial measures are performed where necessary, because carelessness spreads when there is no threat of impending or immediate danger.

Even farther off the mark is the common claim that the application of StT and gaRT (LK usually plays a role in pilot projects only) and the concomitant limit values would be a threat to the industrial base of high-tech nations and meaningful employment for millions of people. Many of the case studies in EEA (2002) suggest that wider use of the precautionary principle can help stimulate both innovation and science, replacing nineteenth-century technologies and the simple science of the first industrial revolution with the "eco-efficient" technologies and systems science of the third.

More important than the negotiation of and the compliance with limit values is, however, to never stop questioning the necessity and/or functional value of pollution-, resource- and exposure-intensive use concepts. It is the exposure that is avoided that is the most compatible with human health, its environments, and nature as a whole. Given this, the observance of the four categories of protection and the three-dimensional rule of environmental hygiene to find and fix goal-specific precautionary limit values are two efficient conceptual tools to sustain environmental hygiene and human health.

Signs and Abbreviations ... ≡ ... "defined/fixed as" or "identical with"

A Class of biogenic/geogenic compounds

ADI See TDI

B Class of functional loads/exposure (compounds with intended function on-site)

BG (Natural) background load/exposure

C Class of nonfunctional loads/exposure (mostly environmental contaminants with no function where detected)

Ea Adverse-effect threshold

F Functional threshold = Threshold of optimized functionality (class B compounds; class C compounds)/threshold of usability (class A) compounds

gaRT Generally accepted rules of technology

LK Level of technical-scientific knowledge

LV Limit value (a maximum concentration fixed by law)

PoD Point of departure = Threshold (dose or concentration) of adverse effect chosen for being extrapolated on humans as part of toxicological evaluation of experimental or epidemiological data to derive an ADI or TDI

REH Three-dimensional rule of environmental hygiene

StT State of technology

T Tolerance threshold

TDI (ADI) Tolerable (acceptable) daily intake of a chemical, mostly given in mg/kg of body mass as considered safe for lifelong exposure

WS Weighting sum

Cross-References

- ▶ Assessment of Background Exposure and Additional Exposure by Human Biomonitoring
- ▶ Ethical Issues in Science: Focus on Regulatory Toxicology
- ▶ Institutionalized Participation in Regulatory Toxicology
- ▶ International Regulation of Toxicological Test Procedures
- ▶ Limit Value Setting in Different Regulatory Areas of Toxicology
- ▶ Nanoparticles and Their Regulation
- ▶ Precaution Principle Versus Danger Prevention in Toxicology
- ▶ Risk Communication: Challenges for Toxicologists and Other Risk Experts
- ▶ Toxicological Risk Assessment in Different Jurisdictions

Notes

1. Culturally conserved or protected loads or exposure (e.g., from natural foodstuffs, certain habitualities of feeding, or from processing of food) occupies a complicated medium position and will not be discussed here.
2. The criteria for whether and how to perceive geogenic/biogenic loads as harmful or harmless refer not only to their scientific evaluation but also to value systems coming from society or cultural history.
3. This “rule of weighting” implies the condition that in this and the following relations, the total weighting sum WS of all signs left and right from the LV is set on 100%, respectively. The differential distribution of the signs left and right the

LV describes the range of expected numerical difference between the respective concentrations of exposure if regulated according to the REH.

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Limit Value Setting in Different Regulatory Areas of Toxicology

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Klaus-Michael Wollin and H. Paul A. Illing

Contents

Abstract

Standards for the protection of human health are important tools used for risk management of chemical substances. They represent the limit value, the maximum level of exposure deemed acceptable or tolerable, under the particular exposure circumstances for which they are set. Usually, there is a formal assessment process by which the standard is set. From a toxicological point of view, limit values reflect a risk characterization for an available data base. Because assessments by individual scientists can differ, limits are usually based on a consensus. Although they must meet a scientific rationale, limit values also have to take into account political considerations, technical feasibility, and economic consequences.

K.-M. Wollin (✉)
Formerly: Public Health Agency of Lower Saxony, Hannover, Germany

H. P. A. Illing
Formerly: Centre for Occupational and Environmental Health, University of Manchester, Manchester, UK

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Limit values · Guidance values · Occupational exposure limits · Contaminated land · Drinking water · Air quality · Environmental health

Introduction

The setting of limit values has to be seen in the context of the general regulatory framework. According to the NAS/NRC (2009) (and IOMC/WHO (2010)) risk assessment/management paradigm, the risk characterization (the qualitative and, wherever possible, quantitative determination of the probability of the occurrence of known and potentially adverse effects of chemical substances under defined exposure condition) is the final stage of the risk assessment. The risk characterization is preceded by the steps hazard identification, hazard characterization, and exposure assessment. The development of alternative regulatory options and the weighing of their economic, social and political consequences are elements of risk evaluation (IOMC), which is the first stage of risk management. The US NAS/NRC report was concerned principally with risk assessment and the EPA does not break down risk management into the three components identified by IOMC, namely, risk evaluation, emission and exposure control, and risk monitoring.

A principle way to regulate harmful substances involves managing exposures in order to prevent their exceeding an exposure considered to represent the maximum acceptable/tolerable level of risk. For the regulator this includes setting and enforcing limit values. In this context, risk assessment and risk management are two related but independent processes. The risk assessment is based exclusively on scientific principles while risk management (and, in particular, the risk evaluation) has to balance problems of socioeconomic costs and benefits, technical feasibility, societal perception, and public policy. The risk management process includes identification of the procedures that should be adopted to control exposure (engineering controls, use of protective equipment, remediation, etc.), the setting of limits, and the enforcement of the procedures and limits. Decoupling of political management and scientific analysis ensures clear responsibilities. It should be emphasized that environmental health policy decisions should be based on established links among emission sources, human exposures, and adverse health effects, i.e., on scientific evidence.

In the narrowest sense limit values are measurable, quantitative thresholds representing uptake at the receptor or site of action within the body for hazardous substances. In practice either the human's body burden of toxic chemical compounds, elements or their metabolites is measured in biological samples (exhaled air, blood, urine, sweat, hair), or by extrapolation from measurements on exposure in various media such as air, water, soil, or food. The limit values have been recommended by the regulatory body established under the appropriate legal framework. Legal limits represent "tolerable" or "acceptable" risks, depending on their definition and the framework within which they are utilized.

The general public uses a very general understanding of the generic term *limit value.* Its scope is extended to guidance values, threshold values, ceiling values, etc., many of which are not enforceable. In contrast, if limit values are treated as values set within a legal framework established by the state, binding thresholds are defined and exceeding these thresholds triggers specific consequences. On the other hand, normally adherence to guidance values (whether from nongovernmental organizations or from government) is voluntary.

The approach used for establishing limit values generally distinguishes between populations. It may also distinguish different levels of protection. A clear definition of the group "at risk" and of the type and level of risk being addressed is one of the most important requirements when setting limits. Thus, health-based limit values can protect different groups of people (to different extents) depending on the circumstances of the exposure; these include:

- Workers
- Consumers
- The general public via environmental exposure (including human health-based standards aimed at protection the environment on the whole or specific compartments (soil, groundwater/surface water, ambient air) within the environment).

The general methodology for establishing health-based limits should be equally applicable both in workplace and non-workplace scenarios. There should be a clear distinction between scientific and other aspects in the practice of setting limit values. Transparency of derivation, flexibility and ease of use, and defined rules for re-evaluation and updating all help to build public acceptance of governmental limit values for the regulation of toxic chemicals. It should be noted that, although apparently different approaches for the risk assessment of chemical substances in the workplace and in other scenarios have emerged on the international and the national level, these differences are due to, inter alia, the standards being for different populations (healthy workers, without children or the elderly and with the possibility of excluding the more susceptible individuals, versus everyone), often with different attitudes to risk, and different exposure scenarios (8 h workplace shifts versus continuous).

The Setting of Occupational Exposure Limits

Occupational Exposure Limit values (OELs) are set by national authorities or national institutions as limits for concentrations of hazardous compounds in the workplace air. Most of the industrialized countries establish and maintain OEL lists that regulate hazardous substance concentration levels to which workers may be exposed via inhalation, ingestion, or skin contact for specified time periods without being at risk over a working lifetime. These limits can be binding or indicative. For workplace airborne exposures to gases, vapors, and particulates, there are three

principal limits in widespread use. They are based on different durations of exposure:

- The 8-h Time-Weighted Average Exposure Limit (TWA): The maximum average concentration of a chemical in air for an 8-h working day and a 40-h week.
- The Short-Term Exposure Limit (STEL): The maximum average concentration to which workers can be exposed for a short period (usually 15 min).
- The Ceiling Value (STEL-C): A concentration that should not be exceeded at any time.

In addition, Biological Exposure Indices (BEIs) represent the body burden, i.e., the concentration of chemicals in the body that would correspond to inhalation exposure at a specific concentration in air. Theoretically, biological effects indices are also possible, but they are unlikely to be set on the grounds that the aim is to prevent harmful effects occurring, and harmful effects are occurring if the measure is one of minimal harm.

Fundamental work to develop a comprehensive approach to setting occupational exposure limits was done by the American Conference of Governmental Industrial Hygienists (ACGIH). The conception of the ACGIH to derive Threshold Limit Values (TLVs) for chemical substances and Biological Exposure Indices (BEIs) is one of the earliest developments aimed at managing workplace exposures. The ACGIH first published Maximum Allowable Concentrations (MACs) from 1946. These were later renamed TLVs, and are republished annually by the ACGIH. TLVs are subject to a health-based view only and are not legally binding. The US Occupational Safety and Health Administration (OSHA), which is a regulatory body, adopts mandatory limits, the Permissible Exposure Limits (PELs), and OSHA is supported in this process by the National Institute for Occupational Safety and Health (NIOSH). NIOSH develops its own health based Recommended Exposure Limits (RELs). Together with ACGIH's TVLS, the RELs of NIOSH contribute to the setting of PELs by the OSHA, however, OSHA makes its own independent judgment regarding the final value of PEL. PELs arise from a comprehensive and well documented rule-making that takes into account significant health risks, sampling and analytical procedures as well as technological and economic feasibility.

Similar approaches to that of ACGIH and NIOSH were adopted by the *Deutsche Forschungsgemeinschaft* (DFG) in Germany (non-enforceable maximum workplace concentration, MAK), the Netherlands and Scandinavia.

The UK Health and Safety Executive (HSE, a regulatory authority with enforcement responsibilities) pursued a dual system of maximum exposure limits (MELs) and occupational exposure standards (OESs), each of which carried different exposure management requirements, until 2005. In 2005, UK's two-OEL system has nominally been replaced by a single OEL system of workplace exposure limits (WELs), in which most of the existing MELs and OELs have been converted to WELs, but the different management approaches previously applicable to MELs and OESs have been maintained using EU classification and labelling requirements to identify which management approach is appropriate. The list of WELs for use with

the Control of Substances Hazardous to Health Regulations 2002 (as amended) (COSHH) is legally binding. Recently, the Health and Safety Executive has approved new and revised WELs that came into force on 17 January 2020 (EH40/2005 Workplace exposure limits, Fourth Edition 2020) (HSE 2020).

On the EU level, there are two principal types of OELs, the so-called Indicative Occupational Exposure Limit Values (IOELVs) and the Binding Occupational Exposure Limit Values (BOELs). IOELVs are health-based nonbinding values, derived from the most recent scientific data available and taking into account the availability of reliable measurement techniques. EU Member States are obliged to take IOELVs into account when implementing national limits for the chemical agents in question. Risk-based BOELs are established for, e.g., carcinogens, mutagens, and airway sensitizers, for which a threshold mechanism is not known. For chemicals for which a BOELV is established at Community level, Member States have to introduce a corresponding national binding limit based on the BOELV. National OELs for both IOELVs or BOELVs may be equal to or lower than the EU OELs. OELs are used to facilitate the regulatory initiatives on occupational exposure limit values for the protection of workers from chemical agent risks, to be set at Union level pursuant to the Chemical Agents Directive (Directive 98/24/EC) and to the Carcinogens and Mutagens Directive (Directive 2004/37/EC).

The European Commission set up the *Scientific Committee for Occupational Exposure Limits to Chemical Agents* (SCOEL) for the work on the scientific evaluation of the health risks posed by exposure to chemical agents in the workplace (Decision 95/320/EC, updated by Decision 2014/113/EU). The major task of the SCOEL has been to advise on the setting of OELs based on scientific data and, where appropriate, propose values (SCOEL 2017). SCOEL's regulatory approach is documented in its *Methodology for the Derivation of Occupational Exposure Limits* (revised 2017). The SCOEL has recommended OELs, which can be supplemented by further notations as:

- Eight-hour time-weighted average (TWA - 8 h)
- Short-term exposure limits (STEL).

If skin penetration of a chemical agent is likely to make a significant contribution to the total body burden and consequently to possible health effects (in general, amounting to 10% or more of the uptake from inhalational exposure at the 8-h TWA), SCOEL assigned a "skin" notation in addition to the establishment of the OEL. A "sensitization (dermal and/or respiratory)" notation may be assigned based upon availability of evidence on either dermal or respiratory sensitization. For respiratory sensitizers, the SCOEL evaluated data on a case-by-case basis. Furthermore, SCOEL may assign a "noise" notation if ototoxicity may occur even at exposures below or close to the established OEL if there is also exposure to excess noise.

The SCOEL aimed to give health-based OELs that can be recommended when the available scientific data suggest that a clear threshold value can be identified as point of departure (PoD) for the adverse effects of the substance in question. For

some adverse effects (in particular genotoxic carcinogenicity, respiratory sensitization, and genotoxicity), it is deemed according to current knowledge that it is not possible to identify thresholds. In these cases, the SCOEL recommended a pragmatic OEL, which is established at levels considered implying sufficiently low risk. Since the late 1990s, SCOEL has developed the concept of "Practical Thresholds" in the derivation of OELs for carcinogens (Bolt and Huici-Montagud 2008). For some carcinogens, health-based OELs have been recommended, while a quantitative assessment of the substance-related carcinogenic risk is made for others. Non-genotoxic carcinogens and/or non-DNA-reactive carcinogens are deemed to have a true threshold associated with a clearly founded NOAEL.

Under certain circumstances, biological monitoring offers advantages over air monitoring in assessing risk to workers´ health. This particularly concerns substances with a skin notation, which are well or predominantly absorbed through the skin in addition to inhalation absorption. Furthermore, biological monitoring should be considered in operations where:

- Oral intake of hazardous substances may be important.
- Workers are exposed to hazardous substances with long biological half-lives.
- An exposure to substances classified as carcinogenic, mutagenic, or toxic for reproduction takes place.
- The hazardous substances are difficult to measure in the air due to strongly fluctuating concentrations.
- Exposure is greater than 8 h per day and 5 days per week.
- An accidental exposure took place.

Where appropriate, SCOEL recommended a *biological limit value* (BLV) which indicate that concentrations equivalent to the BLV are unlikely to result in adverse effects on worker's health. In the case where the available data do not allow to establish a health-based BLV SCOEL may establish a *biological guidance value* (BGV). BGVs are exposure-related guidance values. They represent the upper concentration of the chemical agent or one of its metabolites in any appropriate biological medium corresponding to the 90th or 95th percentile in a defined reference population. Normally, the reference population is a non-occupationally exposed working age population.

When carrying out an assessment of human health effects for the chemical safety assessment under Regulation (EC) No 1907/2006 (REACH), the regulation requires the derivation of a "*Derived No Effect Level" (DNEL)* or *"Derived Minimal Effect Level" (DMEL)* for each relevant human population (e.g., workers, consumers, and humans liable to exposure indirectly via the environment) and possibly for certain vulnerable subpopulations (e.g., children, pregnant women) by the registrant. DNEL or DMEL should be derived for all relevant routes of exposure (inhalation, dermal or oral). Inhalation is usually considered an important potential route of exposure in the workplace. A (generic) maximum "safe" inhalation exposure level can be developed from the appropriate DNEL/DMEL using the recommended (in Guidance from ECHA) standardized procedure and assessment factors. If no OEL is available, the

adequacy of the protective measures used in the workplace can be assessed by comparing the predicted or actual exposure levels with the maximum "safe" exposure level derived from this REACH-based procedure.

In the past, the derivation of an OEL or a DNEL by SCOEL or by using the ECHA guidance quite frequently resulted in numerically different workplace air concentrations for the same substance. Therefore, a "Joint Task Force of the ECHA Committee for Risk Assessment (RAC) and Scientific Committee on Occupational Exposure Limits (SCOEL)" (2017a, b) was engaged in a critical evaluation of scientific aspects and methodologies related to the exposure of chemicals at the workplace to improve mutual understanding of the different approaches and to work towards agreed common scientific approaches including through the further development of existing and new concepts as necessary in relation to workers' exposure to chemicals.

Since January 2019, the scientific evaluation of health effects of hazardous chemical agents in the workplace and derivation of safe levels of occupational exposure (OELs, BVLs) is performed by ECHA's Risk Assessment Committee. The findings of the ECHA/RAC-SCOEL Joint Task Force on alignment of methodologies related to the exposure of chemicals at the workplace, including the inhalation, dermal routes, and in particular carcinogens with or without a threshold mechanism were considered in ECHA's recent "Guidance for preparing a scientific report for health based exposure limits at the workplace" (ECHA 2019).

Health-Based Limit Values for Environmental Contaminants

Air Pollutants

The World Health Organization (WHO) defines air pollution as "contamination of the indoor or outdoor environment by any chemical, physical or biological agent that modifies the natural characteristics of the atmosphere." Effects of air pollutants can impair human health either directly via inhalation exposure or indirectly via atmospheric deposition on edible plants and thus entering the food chain. The dimension of air pollution is a global one. Recent data of WHO (2019) show that over 90% of the world's population lives in places, where air quality exceeds WHO guideline limits.

Outdoor (ambient) and indoor air quality are usually considered separately by WHO. WHO's air quality guidelines (for ambient air quality) were first published in 1987 as "Air Quality Guidelines for Europe" (WHO 1987), followed by the second edition in 2000. WHO emphasizes that these guidelines are not intended as standards. In moving from guidelines to standards, the prevailing exposure levels and environmental, social, economic, and cultural conditions in a country or region should be taken into account. The guideline setting process has been described in detail in the "Guidelines for Air Quality" (WHO 2000). In short, toxic effects are considered to be of two types, threshold and non-threshold. For substances where the critical effect is considered to have a threshold (including non-genotoxic

carcinogenesis for which there is adequate mechanistic data), a *Tolerable Intake* (TI) expressed as airborne concentrations (i.e., μg or mg/m^3) is developed usually on the basis of a NOAEL. A range of safety or uncertainty factors was used but not a rigid framework; these represented the expert judgment of the scientists involved in derivation of WHO's air quality guidelines. The derivation of guidance values for compounds present in other environmental media than air will require the allocation of proportions of the TI to such as air, food, and water, which will be based on sound information on relative exposure via different routes. A default approach, low-dose risk extrapolation, was conducted for carcinogens of IARC classification groups 1 and 2A, and an uncertainty factor approach applied in the case of substances in groups 2B and 3. The mechanism of action was the determining factor for the method of assessment. Hence, it was decided that compounds classified under 1 or 2A could be assessed using uncertainty factors, if evidence for a threshold mechanism of carcinogenicity existed. In contrast, compounds classified under 2B could be assessed by low-dose extrapolation methods, if a nonthreshold mechanism of carcinogenicity in animals was proven.

WHO has revised its air quality guidelines in 2005 for key parameters of contamination (particulate matter, ozone, nitrogen dioxide, and sulfur dioxide). Whereas the previous guidelines (published in 1987 and 2000) concentrated on Europe, the 2005 revision included information from low- and middle-income countries worldwide (WHO 2005). They are designed to offer global guidance on reducing adverse health impacts of air pollution. WHO air quality guidelines are not legally binding, but constitute an important basis for the regulation of air pollution. National air quality *standards* will vary from country to country. They depend on each country's attitude to health risk and its specific approaches to balancing risks to health and technological feasibility. They also take into account economic considerations and political and social factors.

In 2010, WHO proposed its guidelines for selected indoor air pollutants. The substances considered, i.e., benzene, carbon monoxide, formaldehyde, naphthalene, nitrogen dioxide, benzo(a)pyrene, radon, trichloroethylene, and tetrachloroethylene, have indoor sources or sources subadjacent to the building, and are often found indoors in concentrations of health concern (WHO 2010). WHO's guidelines for indoor air quality provide the scientific basis for legally enforceable standards. The WHO guidelines for indoor air quality on household fuel combustion published in 2014 represent a risk-management tool designed to provide guidance and support for people in low- and middle-income countries that still rely on solid fuels burned in inefficient and highly polluting stoves or open fires for cooking and heating. The intervention options, strategies, and recommendations were mainly based on the WHO guidelines for indoor air quality from 2010. WHO's guidelines for indoor air quality on household fuel combustion were the first one developed following the procedures outlined in the WHO handbook for guideline development (WHO 2012 and 2014) that aims at ensuring that WHO guidelines are of high methodological quality and are developed through a transparent, evidence-based decision-making process. It is expected that the coming revision of WHO's global air quality guidelines, started from 2016, will provide updated numerical concentration limits

and, where possible, an indication of the shape of the concentration–response functions for PM10, PM2.5, ozone, NO_2, SO_2, and CO, for short- and/or long-term exposure.

The US *National Ambient Air Quality Standards* (NAAQS) are standards established by the US EPA under authority of the Clean Air Act (CAA) that apply to outdoor air. EPA has set NAAQS for the following principal pollutants (also known as "criteria air pollutants"): Carbon monoxide, lead, nitrogen dioxide, ozone, particulate matter (PM), and sulfur dioxide. The standards are listed in Title 40 of the Code of Federal Regulations Part 50. CAA established two types of national air quality standards. *Primary standards* set limits to protect public health with an adequate margin of safety to allow for the health of vulnerable populations such as individuals suffering from respiratory disorders, children, and the elderly. *Secondary standards* set limits to protect public welfare, including protection against visibility impairment, damage to animals, crops, vegetation, and buildings. When establishing a new NAAQS or revising an existing standard for each criteria air pollutant, US EPA must designate areas as in compliance or not in compliance with the standard. The CAA requires states to develop *State Implementation Plans* (SIPs) to attain and maintain the standards in all areas of the country and a specific plan to attain the standards for areas designated nonattainment.

With the Air Quality Framework Directive 96/62/EC, the European Union (EU) has created the legal framework for future air quality development. Directive 2008/50/EC of 21 May 2008 on ambient air quality consolidated as much existing legislation on objectives for ambient air quality in relation to sulfur dioxide, nitrogen dioxide, and oxides of nitrogen, particulate matter (PM10, PM2.5), lead, benzene, carbon monoxide, and ozone and Directive 2004/107/EC (which was not included in the consolidation) set objectives for arsenic, cadmium, mercury, nickel, and polycyclic aromatic hydrocarbons (PAHs) in ambient air. Under EU law, a *limit value* is legally binding from the date it comes into force subject to any exceedances permitted by the legislation. In contrast, a *target value* is to be attained as far as possible by the attainment date. Target values are less strict than limit values. Member States are required to ensure that limit values are met. Where limit values are not met, EU Member States must take all necessary measures and prepare an *air quality plan* to address the problems identified. EU's legislative instrument to achieve the ambitious 2030 objectives of the Clean Air Programme is the Directive 2016/2284/EU on the reduction of national emissions for the five pollutants sulfur dioxide, nitrogen oxides, volatile organic compounds, ammonia, and fine particulate matter.

Water Quality Criteria and Standards

Quality standards for ground and surface water may reflect either or both ecological criteria and quality criteria for drinking water. Either water resources used as sources of drinking-water, and their related water ecosystems should be protected from pollution or they have to be purified during supply.

The European Union has implemented the Water Framework Directive (EU Directive 2000/60/EC) establishing a framework for Community action in the field of water policy (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2000:327:0001:0072:EN:PDF). Its ultimate objective is to achieve a "good ecological and chemical status" for all Community waters by 2015. The Directive establishes a list of 33 priority substances, including cadmium, lead, mercury, nickel, and its compounds, benzene, PAHs, and DDT for action. The corresponding *Environmental Quality Standards* (EQS) for priority substances and certain other pollutants have been laid down in Annex I of the Directive 2008/105/EC on environmental quality standards in the field of water policy. EQS are derived as an annual average value providing protection against long-term exposure, and maximum allowable concentrations to protect against short-term exposure in order to ensure that the aquatic environment and human health are adequately protected. Generally, groundwater is the most sensitive and the largest body of freshwater and, in particular, is a main source of public drinking water supplies. The Directive 2006/118/EC on the protection of groundwater against pollution and deterioration comprises *groundwater quality standards* for nitrates and active substances in pesticides, including their relevant metabolites, degradation, and reaction products. It also requires Member states to establish threshold values for groundwater pollutants and indicators of pollution on the basis of a minimum list of pollutants and their indicators (arsenic, cadmium, lead, mercury, ammonium, chloride, sulphate, trichloroethylene, tetrachloroethylene, and conductivity [which is indicative of saline or other intrusions]) considering the guidelines outlined in Annex II/Part A.

Section 304(a) (1) of the US Clean Water Act is the legal basis for the development of criteria for ambient water quality for the protection of aquatic life as well as for human health (including organoleptic effects) in the USA. US EPA's (2020) *National Recommended Water Quality Criteria* defines the human health criterion as the highest concentration of a pollutant in water that is not expected to pose a significant risk to human health (https://www.epa.gov/wqc/national-recommended-water-quality-criteria-human-health-criteria-table). The criteria that were revised in 2015 for 94 chemical pollutants consider human health for the consumption of water and organisms or organisms only. The methodology for deriving *Ambient Water Quality Criteria for the Protection of Human Health* has been revised in 2000 with revisions in the assessment of exposure to carcinogens, exposure to noncarcinogens, exposure assessment, and bioaccumulation. For noncarcinogens, the effective EPA guidance on assessing noncarcinogenic effects of chemicals and for the Reference Dose (RfD) derivation should be used. More sophisticated methods are recommended for cancer risk assessment, including identification of the likely mechanism of human carcinogenicity and use of the most appropriate low-dose extrapolation.

WHO's water related activities cover a broad range of activities, including water and drinking water quality and infectious agents, toxic chemicals, and radiological hazards and general aspects of water supply and sanitation as well (WHO 2017). Since 1984, a comprehensive framework, the *Guidelines for Drinking-water Quality* (GDWQ), has been published periodically by the WHO. The last

(4th) edition dates from 2017 incorporating the 1st addendum. Two approaches to derive guideline values are used: one for "*threshold chemicals*" and the other for "*non-threshold chemicals*" (mostly genotoxic carcinogens). In establishing GDWQ, the IARC evaluation of carcinogenic compounds, where available, is taken into consideration. The principles in the derivation of ADIs (acceptable daily intakes) developed by FAO, JECFA, and JMPR have been adopted, where appropriate, in the derivation of TDIs used in developing guideline values for drinking-water quality. GDWQ are kept up-to-date through an ongoing "*rolling revision*" process. Increasingly the preferred approaches for the derivation of TDIs/ADIs for threshold chemicals include the benchmark dose (BMD) or the benchmark dose lower confidence limit (BMDL) and chemical specific adjustment factors. In order to make the distinction with respect to the underlying mechanism of carcinogenicity, compounds that have been shown to be a carcinogen (i.e., chemicals classified in group 1 or group 2A by IARC) are evaluated on a case-by-case basis. The evidence of genotoxicity, the range of species affected, the relevance of the tumors observed in experimental animals to humans and the toxicokinetics of the substance are considered when determining the mode of action, and therefore the approach taken. For carcinogens for which there is evidence to suggest a non-genotoxic mechanism or to suggest that detoxification mechanisms require to be overwhelmed by high doses, guideline values are derived using the threshold chemicals approach. WHO's normal allocation of 20% of the TDI/ADI to drinking-water has changed from the allocation of 10% used in the third edition of the GDWQ. The latter was found to be excessively conservative, and the new value will be incorporated in new guidelines and revisions of existing guidelines.

The current EU binding framework for Member State national standard setting for the quality of *water intended for human consumption at the point of deliver* is contained in the Council Directive 98/83/EC (Drinking Water Directive), last amended by Commission Directive (EU) 2015/1787 of 6 October 2015. The numerical values for chemical parameters in Annex I are generally those of WHO's GDWQ; the opinion of the Commission's Scientific Advisory Committee is a further scientific basis for the quality standards (*parametric values*) in drinking water. The Commission must review Annex I at least quinquennially and has to make proposals for amendments in the light of scientific and technical progress. Member States can include in their own national legislation additional requirements, e.g., regulate additional substances that are relevant or set higher standards but are not allowed to set lower standards as the level of protection of human health should be the same within the whole EU. Temporary derogations from the chemical quality standards as specified in Annex I can be granted, provided it does not constitute a potential danger to human health and provided that the supply of drinking-water in the area concerned cannot be maintained by any other reasonable means.

Under the Safe Drinking Water Act (SDWA), US EPA (2018) evaluates substances that may be found in drinking water by developing Contaminant Candidate Lists contaminants to regulate in drinking water and sets regulatory limits. *Drinking Water Standards* and *Health Advisories* (DWSHA) are issued periodically by US EPA (current tables of drinking water standards and health advisories as of March,

2018). The Health Advisory (HA) Program publishes concentrations of drinking water contaminants at Drinking Water Specific Risk Level Concentration for cancer (10^{-4} Cancer Risk) and concentrations of drinking water contaminants at which noncancer adverse health effects are not anticipated to occur over specific exposure durations: 1-day, 10-day, and Lifetime. The *Lifetime HA* for the drinking water contaminant is calculated from its associated *Drinking Water Equivalent Level* (DWEL), obtained from its Reference Dose (RfD), and incorporates a drinking water Relative Source Contribution (RSC) factor of contaminant-specific data or a default of 20% of total exposure from all sources. *1-day HAs*, *10-day HAs*, and *Lifetime HAs* are not to be construed as legally enforceable Federal standards. In contrast, an enforceable *Maximum Contaminant Level* represents the highest level of a contaminant that is allowed in drinking water. MCLs are set as close as feasible to the *Maximum Contaminant Level Goal* (MCLG) using the best available analytical and treatment technologies and taking cost into consideration.

Soil Values (Contaminated Land)

Land contamination may occur naturally or through anthropogenic activities. A distinction is often made between soil contamination originating from clearly confined sources (local or point source contamination, e.g., abandoned hazardous sites) and that caused by diffuse sources. In general, land contamination and remediation is a newer field of environmental legislation and control is currently mainly through land use planning legislation. Different policies (e.g., on water, waste, chemicals, industrial pollution prevention, pesticides, agriculture) have contributed to preventing land being contaminated. However, as these policies have other aims, they are not sufficient to ensure an adequate level of soil protection. On the European scale, a proposal for a framework Directive (COM (2006) 232) exist which sets out common principles for protecting soils across the EU (European Commission 2006). Taking note that the proposal for a Soil Framework Directive has been pending for almost 8 years without a qualified majority in the Council in its favor, the Commission withdrew this proposal on 30 April 2014. Aspects of EU's soil protection continued to be addressed in the Decision No 1386/2013/EU on a General Union Environment Action Programme to 2020.

Specific soil trigger values have been set in recent times at the national level, notably in Canada, Germany, Netherlands, Switzerland, and United Kingdom. The US EPA developed the Soil Screening Guidance to help standardize and accelerate the evaluation and cleanup of contaminated soils. This guidance provides a methodology to calculate risk-based and site-specific *Soil Screening Levels* (SSLs) for contaminants in soil. To calculate SSLs, the exposure equations and pathway models are run in reverse to back calculate an "acceptable level" of a soil contaminant. For ingestion, dermal, and inhalation pathways, toxicity criteria are used to define an acceptable level of contamination in soil, based on a 10^{-6} individual excess cancer risk for carcinogens and a hazard quotient (HQ) of 1 for noncarcinogens. SSLs are back calculated for migration to ground water pathways using ground water

concentration limits (MCLGs, MCLs, or health-based limits (HBLs) (10^{-6} cancer risk or a HQ of 1, where MCLs are not available). Generic SSLs are not national cleanup standards.

Future Perspectives

Increasingly, scientific quantitative risk assessment succeeds in identifying and reducing uncertainties that are inherent in all stages of the risk analysis. For substances with adverse health effects, alternative methods such as the Benchmark Dose method are being incorporated into the determination of dose-response relationships. These alternatives can reduce the shortcomings of the classical concept of determining tolerable body doses based on a NOAEL or LOAEL. Recent assessments of carcinogenicity are based on the complete analysis of all available biological information, including that on the mechanism of action. This is an improvement on the older risk quantification in the low doses range using the linearized multistage model, which often led to an overestimation of risk. Exposure assessment methods are beginning to allow a more realistic description of exposure. However, better exposure models require an expanded data base. Current issues include the use of multiple "worst case" (or "reasonable worst case") assumptions by regulatory authorities, leading to unrealistically precautionary overall risk assessments. Probabilistic approaches, such as Monte Carlo analysis, yield more realistic overall risk assessments. Emerging issues include approaches to considering the extrapolation to low doses in a sound manner, low-dose effects in toxicology/non-monotonic dose-response, and the development of scientific state of the art approaches to mixtures of chemicals. Future challenges will be the regulatory acceptance of alternatives to animal testing and the use of toxicogenomics data in human health limit value setting.

Cross-References

- ▶ Current Role of the Risk Concept in Regulatory Toxicology
- ▶ Regulation and Mitigation of Greenhouse Gases
- ▶ Risk Characterization in Regulatory Toxicology
- ▶ Risk Evaluation in Regulatory Toxicology
- ▶ Working Areas of Regulatory Toxicology

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Registration and Approval in Regulatory Toxicology

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Thomas Wallenhorst

Contents

Abstract

Consumers are exposed directly or indirectly to all kinds of chemical substances by using products and articles in daily life. Products and articles are regulated in different ways and with various processes depending on the intended uses to ensure the safety of man and environment.

Specific application areas in which consumers are exposed to chemical substances are subject to regulations and approvals. Such areas are chemicals in general for professional and private uses e.g. pesticides, plant protection products, biocides, consumer goods, e.g., cosmetics and toys as well as areas for human

T. Wallenhorst (✉)
Sanitized AG, Burgdorf, Switzerland
e-mail: thomas.wallenhorst@sanitized.com

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health, e.g., medical devices and medicines. These areas are some of the most important and most relevant regulated fields.

The requirements for approval and legally placing the product on the market depend on the intended uses and the appropriate regulation. The key point of every approval process is the evaluation of the potential risk and/or hazard for man and the environment. A risk-benefit analysis has to be performed in order to evaluate if the correlated risk or hazard occurring from the use of the substance, the product, or the article is acceptable. The authorization, registration, or notification of substances, products, or articles for the various application areas is different from country to county and from region to region. The regions with the most advanced and developed registration requirements are Europe, the USA, Canada, and Japan. However, especially Asian countries are implementing more and more regulation processes. Very often are these requirements a mixture of the US and the European system combined with some national demands.

It is the aim to harmonize the requirements and standards for the regulations between the different regions and countries. On an international level, OECD standards bring a certain level of harmonization. In Europe, the EU directives and EU regulation are aiming for a harmonization between the European Member States. Europe is the region with the fastest development in harmonization of the various regulated areas. European regulations and directives of the abovementioned areas are the focus of the following outlines.

Keywords

Chemical inventories · REACH

Chemical Regulations

Relevant regulations:

- Regulation (EC) No. 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No. 793/93 and Commission Regulation (EC) No. 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC
- Regulation (EC) No. 1272/2008 of the European Parliament and of the Council, of 16 December 2008, on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No. 1907/2006

Introduction

Chemicals are regulated through national chemical inventories. The requirements for inclusion of chemicals in the different national chemical inventories vary greatly from country to country. The chemical regulation in the EU went through a dramatic and fundamental change in the past years. The new chemical regulation in the EU is probably the one with the deepest impact on chemical-related industries since the existence of the EU.

REACH: The chemical regulation in the EU is now known as REACH with the official number EC No. 1907/2006. REACH stands for Registration, Evaluation, Authorisation and Restriction of Chemicals. It replaces the former EINECS (European Inventory of Existing Commercial Substances) and the ELINCS (European List of Notified Chemical Substances) lists.

REACH had a tremendous impact on manufactures and importers as well as on the so-called downstream users of chemicals. REACH is a very complex registration system; only the basic points and the frame idea are outlined below.

The REACH regulation became effective on 1 June 2007. The aim of this regulation is to control all manufactured or imported chemicals into the EU in order to improve the protection of human health and environment. The European Chemicals Agency (ECHA), located in Helsinki, is appointed to organize and manage the registration processes and to handle a central database where all necessary data are stored and which is accessible for national competent authorities, professionals, as well as consumers.

REACH affects all chemicals manufactured or imported with a quantity over 1 t/year per producer or importer. The regulation differs between phase-in and phaseout substances. Phase-in substances are chemicals already listed in the EINECS or "no long polymers" as well as chemicals produced 15 years before REACH was in force but were never sold in the market, e.g., internal production. Not under the scope of REACH are polymers (not the monomers); radioactive substances; substances for research; substances in transit; substances which are controlled by other regulations, e.g., medicines for human and animal health and plant protection and biocide active substance, which are considered as registered; substances for food and feed; reimported substance into the EU; as well as substances mentioned in Annexes IV and V, e.g., water, limestone, and natural substances classified as not dangerous.

The whole REACH system is divided into different notification and registration phases with different deadline. In principle, since 1 December 2008, only registered chemicals are allowed on the market. The basic principle is no data – no market. However, if a manufacturer or importer has preregistered the chemical, transitional periods will apply. Only the "phase-in" chemicals are under the scope of the preregistration. The advantage of the preregistration is the extended timelines depending on the amount of chemicals placed on the market (Table 1) and the participation in a so-called SIEF forum. SIEF stands for "Substance Information Exchange Forum." In this forum, different manufacturers and

Table 1 Timelines according to article 23 of the REACH regulation

Amount placed on the market	Timelines
≥ 1,000 t/a	1 December 2010
CMR substances ≥ 1 t/a	1 December 2010
Substances dangerous to the environment ≥ 100 t/a	1 December 2010
Notification Art 7 (4)	1 June 2011
≥100 t/a	1 June 2013
≥1 t/a	1 June 2018

importers can exchange data about the same chemicals and can also share costs for the data needed for the registration.

ECHA has published a list with the preregistered chemicals. The list can be downloaded on the ECHA website (http://echa.europa.eu/web/guest/information-on-chemicals/pre-registered-substances). Under certain circumstances, even if the preregistration phase is over, it is still possible for a late preregistration, e.g., if an importer decides to import a certain chemical.

The requirements for submission of data to a certain timeline depend on the total amount of the chemical placed on the EU market. The higher the amount of chemical manufactured or imported, the more data are required for the registration, and the shorter is the deadline to submit the data. All chemicals above 1 t/year on the EU market are under the scope of the regulation and need to go through the REACH processes. Chemicals classified as carcinogenic, mutagenic, or reproduction toxic (CMR) have special more strict requirements in respect of registration timelines and requested data. For all phase-in and non-phase-in substances, a submission of a technical dossier is required. Besides the physical/chemical properties and toxicological and ecotoxicological properties, the dossier must also include information on classification and labeling, manufacturing, and intended uses as well as instructions for the safe usage of the substance. The technical dossier needs to be submitted via the latest IUCLID (http://iuclid.eu) (International Uniform Chemical Information Database) database software version.

Substances may be recognized as substances of very high concern, so-called SVHCs (substances of very high concern). If a substance is identified as a SVHC, because of its potential negative impacts on human health or the environment, a substance may be included on the authorization list (Annex XIV of the REACH regulation) and become subject to authorization. If a SVHC is placed on the authorization list, the use of this substance needs an authorization by ECHA. Candidates for authorization are included on the candidates list of the SVHC. This list is continually updated and can be downloaded from the ECHA website (https://echa.europa.eu/candidate-list-table). From 1 June 2011, the ECHA must be notified of the presence of SVHCs in articles if the total quantity used is over 1 t/year and the SVHC is present on more than 0.1% of the mass of the object.

An additional important REACH element is the communication along the user downstream. Manufactures as well as users need to communicate and exchange data on the use of the chemical. In this sense, the whole industry is affected from the big chemical industry to the medium-sized and small-sized companies using the chemical.

The REACH regulation is complemented by the GHS (Globally Harmonized System) regulation or also called CLP regulation (EC No. 1272/2008) on classification, labeling, and packaging of substances and mixtures. The CLP regulation became effective on 20 January 2009 and substitutes the EU Directives 67/548/EEC and 1999/45/EC for labeling, classification, and packaging of substances and mixtures.

Information on REACH is available on the ECHA website (http://echa.europa.eu), in particular in the guidance documents. Also national helpdesks provide support for REACH and CLP questions (https://echa.europa.eu/support/helpdesks/). A special situation occurs in Switzerland. Switzerland, not a member of the EU, has an own regulation for chemicals described in the national "Chemikalienverordnung" under the chemical law "Chemikaliengesetz (ChemG)." Switzerland has adapted some parts of the REACH regulation, e.g., the CLP regulation, but Switzerland has not adapted the general REACH regulation, and there are essential differences between the Swiss legislation and the REACH regulation. Information is available on the Swiss Chemical website (https://www.bag.admin.ch/bag/de/home/das-bag/organisation/direktionsbereiche-abteilungen/direktionsbereich-verbraucherschutz/anmeldestelle-chemikalien.html).

Pesticides

Pesticides are essential for agriculture and high hygiene standards in our society. Pesticides are chemical compounds intended to kill, repel, and control pests, to protect crops before and after harvest, to destroy weeds preventing their growth, as well as to influence and preserve plant products. Pesticides cover a broad range of specific protection products like insecticides, acaricides, herbicides, fungicides, plant growth regulators, rodenticides, biocides, and veterinary medicines.

The EU regulates pesticides strictly to ensure safety of humans and environment and to ensure the efficacy of the used products.

The EU established an approval and authorization system on pesticides, especially on plant protection products and biocidal products in a two-step approach:

Step 1.
The Commission approves the active substances contained in the products.
Step 2.
EU Member States authorize the products on their territory and ensure compliance with EU rules.

Plant Protection Products

Relevant regulations:

- Regulation (EC) No. 1107/2009 of the European Parliament and of the Council of 21 October 2009, concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC

The Plant Protection Products Directive was introduced in 1991 with the Council Directive 91/414/EEC, regulating the placing of plant protection products on the EU market. The directive lays down rules and procedures for approval of active substances at EU level and for the authorization at Member State level of plant protection products (PPPs) containing these active substances. The Directive 91/414/EEC was replaced by Regulation (EC) No. 1107/2009.

The regulation intents to ensure a high level of human, animal, and environmental protection and to provide clear rules to make the approval process for plant protection products more effective.

A plant protection product contains one or usually more active substances. Before companies can use an active substance in a plant protection product, the active substance needs to be approved by the responsible EU Committee. The EU Regulation 1107/2009 regulates the approval and use of plant protection products and active substances. An active substance will only be approved if the criteria according to Annex II No. 2 and No. 3 are fulfilled. The criteria consider efficacy of the active substance, available analytical methods, and effects on human health and environment. The approval of an active substance is not equivalent with the approval of a plant protection product (formulation). The plant protection products need to be also approved by the concerned Member States.

To find out which active substances are approved in the European Union, a database can be consulted on the website of the European Commission (http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database). For each substance, there is a reference to the EU legislation, including the relevant toxicological information and the maximum residue levels in food and feed.

More general information concerning active substances and plant protection products and different guidance documents can be found on the EU website (http://ec.europa.eu/food/plant/protection/evaluation/index_en.htm).

In correlation to Regulation (EC) No. 1107/2009, two related topics need to be mentioned: one is the Regulation (EC) No. 396/2005 which regulates the residues of pesticides in food and the Directive 2009/128/EC which regulates the sustainable uses of pesticides.

In Switzerland, not an EU Member State, plant protection products are regulated by the “Pflanzenschutzmittelverordnung” (PSMV). This regulation has many similarities to the EU regulation. Further information is available at the website of the BAG (Bundesamt für Gesundheit) (http://www.admin.ch/ch/d/sr/c916_161.html).

Biocides

Relevant regulations:

- Regulation (EU) No. 528/2012 of the European Parliament and of the Council of 22 May 2012, concerning the making available on the market and use of biocidal products

Biocides are essential for the health and hygiene standards in our societies. Biocides are used in various application fields like disinfections and pest control or as preservatives to protect perishable materials. Biocides are regulated in the EU under the Biocidal Products Regulation (BPR) EU No. 528/2012, which replaced the initial Biocidal Products Directive (BPD) 98/8/EC. Before the BPD, biocides were regulated individually by the national Member States with different registration or notification systems or were not regulated at all. With the BPD (98/8/EC), the EU tried to implement a harmonized framework on biocidal products.

Essential for understanding of the authorization processes is the differentiation between an active substance, a biocidal product, and a treated article. A chemical or a microorganism that has an action on or against harmful organisms is defined as an active substance. Formulations with an active substance are defined as biocidal products, which are usually used for various biocidal purposes. The definition, according the Biocidal Products Regulation (EU) No. 528/2012, of a biocidal product includes all substances or mixtures containing or generating active substances with the intention to destroy, deter, render harmless, prevent the action, or exert otherwise a controlling effect on any harmful organism by any means other than mere physical or mechanical action. A treated article is a substance, mixture, or article which has been treated with, or intentionally incorporates, one or more biocidal products.

The BPR determines a two-step approach for the authorization of a biocidal product. In the first step, the approval of the active substances takes place at EU level. The assessment of the single active substance for the intended uses, the so-called product type (PT), has been allocated to the competent authorities of an EU Member State. The appointed EU Member State reports the results of the evaluation, which is then discussed at the competent authority meetings. It is the task of the responsible EU Committee to decide on the inclusion in the positive list for active substances (union list). In the second step, the subsequent authorization of the biocidal product is on the single EU Member State, meaning that an application for authorization needs to be submitted to the Member State in which the biocidal product is planned to be market. Also a union authorization, covering all EU Member States, is a possible path. In this case the ECHA takes an administrative and organization role.

One key point of the BPR is the assignment of an active substance to 1 of the 22 different application fields for the intended uses outlined in the 22 product types (PTs) as described in Annex V of the BPR.

There are four main groups of PTs, which are further divided in subgroups adding up to 22 PTs. The four main PTs are:

- Disinfectants and general biocidal products, e.g., human hygiene, private and public health area, veterinary hygiene, food and feed area, and drinking water disinfectants
- Preservatives, e.g., in-can or film preservatives; wood preservatives; preservatives for leather, plastics, rubber, and masonry; preservatives for liquid-cooling and liquid-processing systems; slimicides, e.g., for paper production; and metal-working fluid preservatives, as well as bad odor protection (e.g., on textiles)
- Pest control, e.g., rodenticides, avicides, molluscicides, piscicides, insecticides, repellents, and attractants
- Other biocide products, e.g., preservatives for food or feedstock and antifouling products (e.g., for marina uses)

With the assignment to a PT, a clear intended use and application field is defined. Any uses in other applications are clearly separated. Other uses are not under the scope of the BPR if these intended uses are controlled by their own regulations, e.g., pharmaceuticals for human and animals, plant protection, cosmetic, medical devices, and food and feed.

Active substances already placed on the market before 14 May 2000 are considered as "existing active substances," active substances placed on the market after that date are considered as "new active substances." Manufactures, formulators, or importers could notify active substance which they wanted to support until 28 March 2002. All identified existing active substances are listed in Annex 1 of Regulation EU 1451/2007. All notified active substances are listed together with the assigned PT in Annex 2 of the Regulation 1451/2007. The EU Commission updates the ongoing evaluation and decisions on the approval or non-approval of the active substances for the assigned PTs. The current status can be checked on the ECHA web page for biocides (https://echa.europa.eu/information-on-chemicals/biocidal-active-substances).

New elements in the BPR are also exclusion and substitution criteria for active substances. If a substance meets the exclusion criteria, the substance will not be approved. Exclusion criteria are, e.g., carcinogens, mutagens, and reprotoxic substances category 1A or 1B according to CLP classification; endocrine disruptors; persistent, bioaccumulative, and toxic (PBT) substances; as well as very persistent and very bioaccumulative (vPvB) substances. Active substances falling under the substitution criteria will be candidates for substitution during the approval processes.

Under the BPR, the authorization of the biocidal product is still mainly at the national competent authority level. That means if an active substance is approved and a company is using the approved active substance for a biocidal product, the company needs to apply for authorization in a Member State. However, if an authorization is granted in a Member State, the applicant has the possibility to ask for recognition of the authorization in another Member State. This so-called mutual-recognition procedure can be performed in sequence or in parallel.

In addition to the mutual recognition of an authorization, a union authorization for all Member States is now possible under the BPR. The union authorization is organized by the ECHA and is only possible for single PTs currently.

A new tool of communication is a database for biocidal products, the so-called Register for Biocidal Products (R4BP). The R4BP database will be maintained by ECHA and is used as a submitting and exchanging platform between applicants, ECHA, Member States, competent authorities, and the European Commission.

Another main new subject under the BPR is the regulation of so-called treated articles. Treated articles are articles which have been treated with or intentionally incorporated one or more biocidal products. Treated articles do not fall under the definition of a biocidal product and do not need an authorization. However, it will be required to label the article with certain information, e.g., that the article was treated with a biocidal product and the name of the active substance the article includes. Treated articles are widely spread in articles used by consumers in daily life, e.g., treated wood, plastic, and leather products, as well as in technical textiles and apparel, furniture, and building material. The correlation between active substances, biocidal products, and treated articles is shown in Fig. 1.

Under the BPR transitional periods still exist and will continue to exist especially for active substances, which are still in the evaluation phase for inclusion in the union list. The evaluation of active substances is planned to be finalized by 2024 (EU Regulation 736/2013). Existing active substances, still in the evaluating phase, can be marketed until a decision is taken. This situation revels in transitional period for those substances. Biocidal products containing substances still in the evaluation

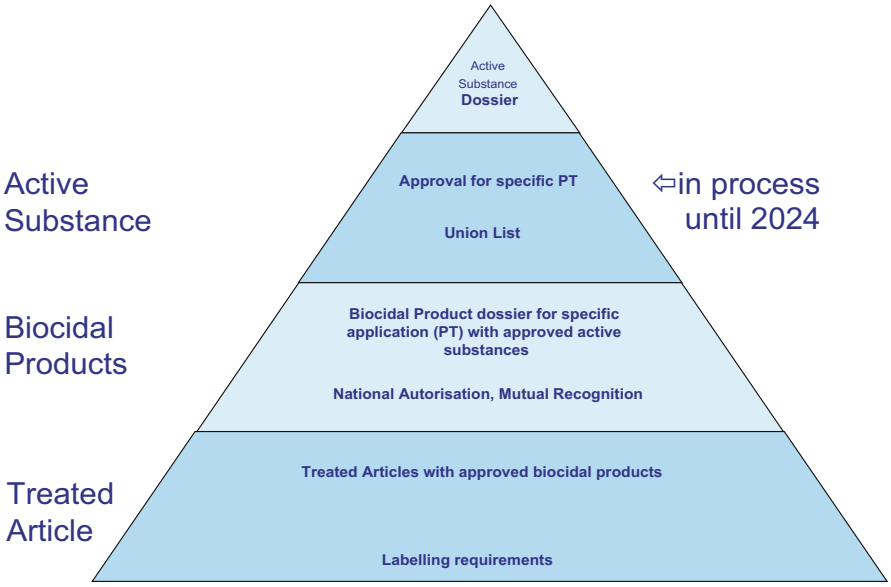

Fig. 1 BPR, correlation between active substances, biocidal products, and treated articles (*PT* product types)

phase are subject to national regulations. The nation regulations are very different from Member State to Member State. In some Member States, a simple notification is requested, e.g., Germany. In other Member States, a complete BPR dossier is required, e.g., in the Netherlands, or a time-consuming complex notification and registration system was implemented, e.g., in Belgium.

It needs to be mentioned that active substances registered or in the evaluation phase under the BPR are excluded from the REACH requirements. These substances are considered as registered. However, other chemical substances included in the biocidal products, e.g., solvents, stabilizers, and emulsifiers, are still subjects to REACH.

Information about biocides is available on the EU Commission biocide website (https://echa.europa.eu/regulations/biocidal-products-regulation) or on the website of the national authorities usually under the chemical section. Switzerland, not a member of the EU, adapted fully the EU legislation for biocides and has implemented the "Biozidprodukteverordnung" (VBP) under the chemical law. Information is available on the chemical website of the BAG. Therefore, Switzerland has the same status as regular EU Member States and can act as a RMS (Reference Member State) as well as a CMR (Concerned Member State) in the approval process under the BPR in the EU (https://www.anmeldestelle.admin.ch/chem/de/home/themen/pflicht-hersteller/zulassung-biozidprodukte.html).

Food Contact Materials

Relevant regulations:

- Regulation (EC) No. 1935/2004 of the European Parliament and of the Council of 27 October 2004, on materials and articles intended to come into contact with food and repealing Directives 80/590/EEC and 89/109/EEC
- Commission Regulation (EU) No. 10/2011 of 14 January 2011, on plastic materials and articles intended to come into contact with food
- Commission Regulation (EC) No. 2023/2006 of 22 December 2006, on good manufacturing practice for materials and articles intended to come into contact with food
- Commission Regulation (EC) No. 450/2009 of 29 May 2009, on active and intelligent materials and articles intended to come into contact with food
- Commission Regulation (EC) No. 282/2008 of 27 March 2008, on recycled plastic materials and articles intended to come into contact with foods and amending Regulation (EC) No. 2023/2006
- Commission Directive 2007/42/EC of 29 June 2007 relating to materials and articles made of regenerated cellulose film intended to come into contact with foodstuffs
- Council Directive of 15 October 1984 on the approximation of the laws of the Member States relating to ceramic articles intended to come into contact with foodstuffs
- Further substance specific regulations

Food contact materials are intended to come into contact with food, e.g. packaging material, containers, cutlery and dishes, material in contact with water for human consumption, but it does not cover fixed public or private water supply equipment. The regulation of food contact materials in the EU is a complex system of regulations and directives. The frame Regulation EU No. 1935/2004 describes the general requirements for all food contact materials. In principle, material intended to come into contact with food shall not release chemicals into food in unacceptable quantities. Furthermore, the material shall not change the food in composition and in quality, and it shall not impact taste, smell, or appearance of the food.

The overall migration limit for plastic materials is set to 10 mg of substances/dm^2 of food contact surfaces, which is equivalent to 60 mg/kg foodstuff for all substances that can migrate from the contact material into food. Specific migration limits (SML) are also established for individual substances based on ADI (acceptable daily intake) or TDI (tolerable daily intake) values established by the European Food Safety Authority (EFSA). At EFSA's website opinions can be searched for substances to be used in food contact materials (http:/www.efsa.europa.eu/en/topics/topic/food-contact-materials). Regulation EU No. 10/2011 regulates plastic materials and articles intended to come into contact with food, replacing the EU Directive 2002/72/EC, and includes a list of approved substances (Annex I) for food contact materials. This regulation also outlines the test methods and conditions for the measurement of migration.

An overview of the related regulations and directives for food contact materials is outlined and illustrated in Fig. 2.

Frame legislation:

Regulation EC No. 1935/2004: Frame regulation on materials and articles intended to come into contact with food
Regulation EC No. 2023/2006: Good manufacturing practice

Legislation on specific materials:

Regulation EU No. 10/2011, updated by Regulation EU No. 1282/2011: Plastic materials and articles intended to come into contact with food
Regulation EC No. 450/2009: Active and intelligent materials and articles intended to come into contact with food
Regulation EC No. 282/2008: Recycled plastic material and articles intended to come into contact with food
Directive 2007 /42/EC: Materials and articles made of regenerated cellulose film intended to come into contact with food
Directive 84/500/EEC, amended by Directive 2005/31/EC: Sets migration limits for cadmium and lead which might be released from decoration or glazing

Legislation on specific substances:

Regulation No. 1895/2005/EC: Restriction of use for certain epoxy derivatives

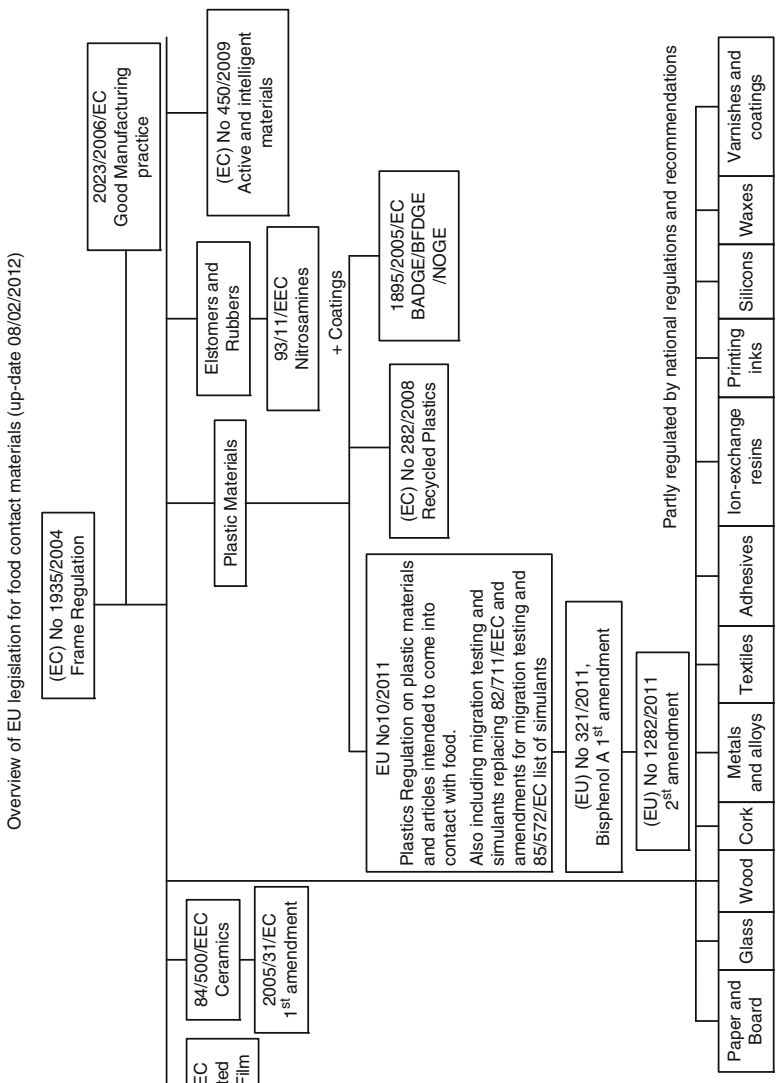

Fig. 2 Overview of EU legislation on food contact material. (Source: References of the European and National Legislations, European Commission 2012)

Regulation EU No. 321/2011: Restriction on bisphenol A use in plastics for infant feeding
Regulation EU No. 284/2011: On import procedures for polyamide and melamine plastic kitchenware from China and Hong Kong
Directive 93/11/EEC: Release of N-nitrosamines and N-nitrosatable substances from rubber teats and soothers

Further information can be found on the EU website (http://ec.europa.eu/food/food/chemicalsafety/foodcontact/index_en.htm).

Toys

Relevant regulations:

- Directive 2009/48/EC of the European Parliament and of the Council of 18 June 2009, on the safety of toys

Toys are a source for exposure of chemicals to the consumer. Therefore, toys must meet high safety standards to ensure the safety of consumers in general and for children in specific. Toys are regulated by the "Toy Safety Directive" 2009/48/EC, which replaces the old Directive 88/378/EEC.

The directive lays down the basic safety criteria toys must meet before being placed on the market. Technical details are described in technical harmonized standards which are suitable to ensure the safety of toys, e.g., Standard EN 71-2 for flammability or Standard EN 71-3, and migration of certain elements, e.g., metals.

Toys are not subject to registration processes. However, toys underlie the conformity assessment in which the conformity with the applicable safety standard needs to be confirmed. The conformity is identified by the CE mark on toy articles.

Details and further information can be found on the EU website (http://ec.europa.eu/growth/sectors/toys/safety/legislation_en).

Cosmetics

Relevant regulations:

- Regulation (EC) No. 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products

Cosmetic products are products only intended to be used for contact with the various external parts of the human body, e.g., epidermis, hair system, nails, lips, or teeth, and the mucous membranes of the oral cavity with the intention of cleaning

them, perfuming them, changing their appearance, and/or correcting body odors and/or protecting them or keeping them in good condition.

In comparison with the already discussed previous regulated areas, substances used for cosmetic products are intended for the exposure to consumers. Cosmetic products are regulated by the EU Regulation No. 1223/2009. This Cosmetic Regulation came into force in January 2010 and was effective on 1 July 2013, replacing the older Cosmetic Council Directive 76/768. The general structure of the former Cosmetic Directive is also reflected in the new Cosmetic Regulation. There is no registration requirement for cosmetic products before placing them on the market. However, substances included in cosmetic products are regulated depending on their function. The Cosmetic Regulation has included positive and negative lists of substances for specific uses.

On the negative list are substances that are not allowed in cosmetic products, e.g., CMR substances. On the positive list are substances for the specific intended use as preservatives, UV filters, and dyes with concentration limits. An update of the different lists is done regularly by amendments to the regulation by Commission Regulations.

Substances, which are not regulated by negative or positive lists, are permitted as ingredients in cosmetic products as long as they are safe for consumers. Therefore, a key element of a cosmetic product is the dossier with the safety assessment that needs to be available on demand for the competent authority.

Responsible for the safety of cosmetic products is the importer, manufacturer, or distributor placing the product on the market.

More information is provided at the EU website for cosmetics (http://ec.europa.eu/consumers/sectors/cosmetics/index_en.htm). Switzerland, not a Member State of the EU, adapted the EU Cosmetic Regulation 1223/2009. The new Swiss Cosmetic Regulation came into force by 01 May 2017 (https://www.admin.ch/opc/de/classified-compilation/20143407/index.html).

Medical Devices

Relevant regulations:

- Regulation (EU) 2017/745 of the European Parliament and of the Council of 5 April 2017 on medical devices, amending Directive 2001/83/EC, Regulation (EC) No. 178/2002 and Regulation (EC) No. 1223/2009 and repealing Council Directives 90/385/EEC and 93/42/EEC
- Regulation (EU) 2017/746 of the European Parliament and of the Council of 5 April 2017 on in vitro diagnostic medical devices and repealing Directive 98/79/EC and Commission Decision 2010/227/EU

Medical devices are instruments, apparatuses, implants, in vitro reagents, or related articles that are used to diagnose, prevent, or treat disease or other conditions and do not achieve their purposes through chemical action within or on the body.

Medical devices act by physical, mechanical, or thermal means. Medical devices vary greatly in complexity and application. They cover a vast range of equipment from simple tongue depressors to hemodialysis machines and pacemakers.

Rules that relate to safety and performance of medical devices were harmonized in the EU. The core legal framework consists of three basic directives:

Directive 93/42/EEC regarding medical devices (MDD)
Directive 90/385/EEC regarding active implantable medical devices (AIMD)
Directive 98/79/EC regarding in vitro diagnostic medical devices (IVDD)

The aim of a harmonized Medical Device Directive in the EU is to ensure a high level of protection of human health and safety. These three main directives have been supplemented and amended over time by several modifying and implementing directives. Directive 2007/47 EC introduced the last bigger technical revision.

Two new revisions of the regulatory framework for medical devices in the EU were adopted by the EU Parliament 5 April 2017.

- Regulation(EU) 2017/745 (regulation on medical devices, MDR)
- Regulation (EU) 2017/746 (regulation on in vitro diagnostic medical devices, IVDR)

The new regulations came into force on 26 May 2017 and replace the old directives in several graduated transition periods. Several elements of the new rules come into force after 6 months. After the transitional period of 3 years, the regulation on medical devices will be completely mandatory (May 2020), and after 5 years, the regulation on in vitro diagnostic medical devices (May 2022) will be mandatory.

The new regulations shall improve the safety for patients and ensure a high quality and safety level of the products.

The main changes and improvements of the new rules among others are:

- Stricter ex ante control for high-risk devices
- Reinforcement of criteria for the designation and processes for oversight of Notified Bodies.
- The inclusion of certain aesthetic devices
- The introduction of a new risk classification system for in vitro diagnostic medical devices
- The introduction of an implant card
- The reinforcement of rules on clinical evidence
- Strengthening of post-marketing surveillance
- Improvement of coordination between EU countries in the field of vigilance and market surveillance

Medical device regulations are based on a compliance system. The responsibility for safety and compliance with the EU regulations is on the side of the medical device manufacturer or importer. Manufacturers guarantee the authorization of

medical devices through a Declaration of Conformity. Medical devices belonging to class I can be marketed by self-certification of the manufacturer. Certification of higher-risk products (class IIa, IIb, or III) must be verified by a Certificate of Conformity issued by a Notified Body. A Notified Body is a public or private organization that has been accredited to validate the compliance with the according regulations. All medical devices must be identified with the CE mark.

Medical devices are divided into different classes based on their design complexity, their use characteristics, and their potential for harm if misused. A combination of medical devices and drugs, so-called combination products, needs to follow a special regulatory process before being marketed.

The classification of medical devices in the European Union is outlined in the Regulation (EU) 2017/745 or in the former Directive 93/42/EEC. There are basically four classes, ranging from low risk, class I, to high risk, class III:

Class I (e.g., wound bandages)
Class IIa (e.g., surgical suture)
Class IIb (e.g., lung ventilator)
Class III (e.g., pacemaker)

The European classification depends on rules that involve the medical device duration of body contact, invasive character, use of an energy source, effect on the central circulation or nervous system, diagnostic impact, or incorporation of a medicinal product.

More detailed information and guidance (MEDDEV Guidance) are available on the EU website:

References:
https://ec.europa.eu/growth/sectors/medical-devices_en
http://ec.europa.eu/growth/sectors/medical-devices/regulatory-framework
https://ec.europa.eu/growth/sectors/medical-devices/guidance_en

Medicines

Relevant regulations:

- Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the community code relating to medicinal products for human use
- Regulation (EC) No. 726/2004 of the European Parliament and of the Council of 31 March 2004 laying down community procedures for the authorisation and supervision of medicinal products for human and veterinary use and establishing a European Medicines Agency

Medicine products, also called medicinal products or pharmaceuticals, are chemical substances that achieve their principal action by pharmacological, metabolic,

or immunological means. The mode of action is different than for medical devices. Medicines are intended to have an effect on humans and animals. An exposure of the chemical substance on humans or animals is intended. The authorization of medicines is the most complex and most expensive regulated field compared to other regulated areas.

To ensure a high safety standard and an acceptable risk-benefit ratio, medicines need to pass a complex authorization process.

The requirements for medicines are basically very similar in all countries with an authorization system for medicines and are very much harmonized in Europe, North America, and Japan. The basic requirement for an authorization of a medicine is the acceptable pharmaceutical quality, pharmaceutical efficacy, safety for the patient, and an acceptable risk-benefit ratio.

The medicine must have an acceptable quality according to pharmaceutical rules. The guidelines and rules of pharmaceutical quality are described, e.g., in the pharmacopeia monographs. The pharmaceutical quality covers the composition of a medicine, the manufacturing processes, and the quality control of the raw materials, the intermediates, and the final product as well as storage stability studies. The manufacturing processes need to comply with the rules for "good manufacturing practice" (GMP).

The efficacy of a medicine is a basic requirement for the authorization. The efficacy is the intended effect to heal diseases or improve the health of a patient. Safety can only be considered in relative terms. All medicines carry a certain degree of risk and could cause problems in specific circumstances. The safety of a medicine needs to be demonstrated by nonclinical and clinical studies. In order to obtain a complete safety profile of the medicine, observations of the continuously collected "pharmacovigilance" reports are included in the safety evaluation of the competent authorities. In this way also long-term effects, which could not be discovered during the clinical studies, can be recognized. Pharmacovigilance is the pharmacological science relating to the detection, assessment, understanding, and prevention of adverse effects particularly long-term and short-term side effects of medicines (http://apps.who.int/medicinedocs/en/d/Js4893e/). The risk-benefit ratio reflects the possible benefit of the medicine for the human or the animal in relation to the possible risks, which could occur, e.g., possible side effects. The benefit risk is also constantly under observation using the pharmacovigilance reporting.

For the authorization of a medicine, all necessary data and studies need to be submitted to the competent authorities for evaluation. In order to harmonize the submitted data, a standard application format dossier was developed by the ICH (International Conference on Harmonization). The standard application format is known as the Common Technical Document (CTD). The CTD dossier consists of five modules, which include all the necessary information.

Module 1 is about regional and specific information, not part of the CTD dossier.
Module 2 includes an overview and summary of modules 3, 4, and 5.
Module 3 includes the quality part, describing manufacturing and analytics of the medicines.

Module 4 includes the preclinical pharmacological and toxicological studies.
Module 5 includes the clinical studies.

Detailed information is available on the ICH website (http://www.ich.org).

The approval procedures are regulated by national regulations, international regulations, and international mutual-recognition procedures. In the EU, the authorization of medicines was regulated in the beginning by Directive 65/65/EEC, which has been replaced by the Directive 2001/83/EC; in addition EU Regulation No. 726/2004 describes the procedure for centralized procedures authorization. The directive is integrated in the different national laws for the authorization of medicines.

The regulation in the EU offers several different ways of how a company can apply for the authorization of medicines. The national procedure was the only way in the EU until 1995. Since then the European procedure gained importance, and the national procedures were preplaced by the European ones. Nowadays the national procedure is only possible in one Member State. Multiple national procedures are not possible anymore. Nevertheless, national competent authorities still play a major role in the authorization process.

The centralized authorization procedure for human and veterinary medicine is managed by the EMEA (European Medicines Agency) in London, and the EU Commission grants the authorization. The authorization is valid in all EU Member States as well as in Iceland, Lichtenstein, and Norway. The centralized procedure is mandatory for human medicines for the treatment of cancer, diabetes, HIV/AIDS, neurodegenerative diseases, autoimmune and other immune dysfunctions, and viral diseases; veterinary medicines for use as growth or yield enhancers; medicines derived from biotechnology processes, e.g., genetic engineering, gene therapy, and somatic cell therapy or tissue-engineered medicines officially designated "orphan medicines" (medicines used for rare human diseases).

Decentralized procedure is used when an authorization is intended simultaneously in several EU Member States if the medicine does not already have an authorization in an EU Member State.

Table 2 Different ways of authorization in the EU

	Centralized procedure	Decentralized procedure	National procedure	Mutual- recognition procedure
Procedure	Authorization in all EU Member States	Authorization in several EU Member States simultaneously	Only national authorization	Authorization on the base on an existing authorization in another Member State
Competent authority (CA)	EMEA	EMEA and national CA	National CA	National CA
Validity	All EU Member States including Iceland, Norway, Lichtenstein	All Member States in which the authorization is accepted	In the concerning national state	In the Member States who accepted the application

Mutual-recognition procedure needs to be used if a national authorization has already been granted and additional authorization in other Member States is intended (Table 2).

Further and more detailed information is available on the websites of the EMEA (http://www.ema.europa.eu, https://ec.europa.eu/health/authorisation-procedures_en) and the national competent authorities responsible for medicinal products.

Cross-References

- ▶ REACH and CLP: Its Role in Regulatory Toxicology
- ▶ Regulation of Dental Materials
- ▶ Risk Minimization in Drug Development: Regulatory Aspects

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Health Hazard Classification and Labelling 68

Herbert Desel, Kathrin Begemann, Ronald de Groot, and Pieter Brekelmans

Contents

Abstract

Consumers are informed about the hazards and safe use of a product on the product label by, e.g., hazard pictogram(s) and other label elements. The hazard classification

Disclosure statement: The authors have been active participants in the European Association of Poisons Centres and Clinical Toxicologists (EAPCCT) Working Group on Poisons Centres' Activities/European Regulatory Issues, and as poisons centre experts, they take part in discussions on harmonizsation of product notification for poisons centres with the European Commission, the European Chemicals Agency (ECHA), industry, and other stakeholders and thus include poisons centres' perspectives on the topic.

H. Desel (✉)
Department Exposure, Unit 32, Exposure Assessment of Hazardous Products, German Federal Institute for Risk Assessment, Berlin, Germany
e-mail: Herbert.Desel@bfr.bund.de

K. Begemann
Department Exposure, Unit Exposure Assessment of Hazardous Products, German Federal Institute for Risk Assessment, Berlin, Germany
e-mail: Kathrin.Begemann@bfr.bund.de

R. de Groot · P. Brekelmans
National Poisons Information Center (NVIC), University Medical Center Utrecht, GA, Utrecht, The Netherlands
e-mail: R.degroot-5@umcutrecht.nl; P.Brekelmans@umcutrecht.nl

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criteria and communication elements for substances and mixtures are worldwide harmonized by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN-GHS). In the European Union, the UN-GHS is implemented by the Regulation on Classification, Labelling and Packaging of Substances and Mixtures (EC) No. 1272/2008 (CLP Regulation). Professional users have access to more comprehensive information (including safe use scenarios) in the Safety Data Sheets (SDS) as required by the REACH Regulation (EC) No. 1907/2006, Article 31.

In case of a medical incident, i.e., an unsafe exposure or an exposure with poisoning concern, product labelling information and SDS are the starting points for adequate medical management, but more detailed information is needed in many cases.

Keywords

Classification · Labelling · Hazardous substances and mixtures · CLP Regulation · REACH Regulation · Health hazard · European chemicals legislation · Safety Data Sheet · Poisons centres · Communication

Introduction

Several regulations of the European Union (EU) aim to ensure a high level of protection of human health from the risks that can be posed by chemical substances on the EU market. Classification and labelling of hazardous substances and mixtures are important in communicating the hazards and providing the basis to describe and plan for safe use. Furthermore, classification supports the poisoning risk assessment if persons have been exposed in an unsafe way. In the past, different systems for the classification and labelling of chemicals were used around the world. A substance or mixture could be classified as hazardous in one country and not classified as hazardous in another country. This led to diverse problems on the industrial or professional side but also to misunderstandings among consumers. To harmonize hazard classification criteria and communication elements worldwide, the United Nations Globally Harmonized System (UN-GHS) of Classification and Labelling of Chemicals (UNCED 1992; UNECE 2003) was developed. The Regulation on Classification, Labelling and Packaging of substances and mixtures (EC) No. 1272/2008 (CLP Regulation) implements UN-GHS in the EU (EC 2008). CLP Regulation entered into force on 20 January 2009 and became valid for classification, labelling, and packaging of substances on 1 December 2010 and for mixtures on 1 June 2015.

Due to the Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (EC) No. 1907/2006 (REACH), industry is responsible for assessing and managing the risks of chemicals and for gathering all relevant substance information for registration at the European Chemicals Agency (ECHA). REACH (see Cross-References, below) incorporates requirements for the Safety Data Sheet (SDS), an important document informing professionals on safe use of a substance or mixture ("product").

Health Hazard Classification

The three elements structuring the CLP Regulation are hazard classification, labelling, and packaging. An overview of classification and labelling is presented here.

The classification describes substances or mixtures in terms of their physical, health, or environmental hazards. The manufacturers, downstream users (e.g., formulators, professional end users), and importers are responsible for providing appropriate classification for most substances and all mixtures. As an exception, substances with fixed classification according to Annex VI have to be classified as indicated in this Annex. To perform the classification, companies have to identify all hazard-related data available and evaluate its quality (weight of evidence). The CLP Regulation defines the criteria according to which the hazardous properties of a substance or a mixture are determined and offers the following options for obtaining the relevant information:

- Animal experiments with the substance or mixture with regard to its hazardous properties
- Other animal experiment-free methods for substances such as (Q)SAR
- Evaluation by applying a weight of evidence determination using expert judgment, including experience on humans, such as epidemiological data, data from accident databases and occupational data, all new scientific information, and any other information obtained from internationally recognized chemical programs

For mixtures, data indicating the health hazard profile is only rarely available. If no hazard data on the mixture is available, then procedures listed in Annex I of the CLP Regulation can be used to evaluate its hazard. Furthermore, official calculation methods allow deduction of the mixture classification from classification of its ingredients if insufficient data were available for hazard evaluation of the mixture.

The framework of the CLP health hazard classification (CLP, Annex I, Part 3) is based on ten health hazard classes, describing the quality of action of the hazard in focus (e.g., "acute toxicity," "carcinogenicity"). The quantity measure (strength or potency) of a hazard quality is described using numeric hazard categories, where higher category numbers (1 to a maximum of 4) indicate lower strength or potency. For some hazard classes, only one category is defined, while for some classes, category 1 is subdivided into 1A, 1B, and 1C. An overview of CLP health hazard classes and categories is presented in Table 1.

Health Hazard Labelling

The second element within the CLP Regulation is the labelling of substances and mixtures. The labelling is used for hazard communication and is based on hazard classification. It informs the user about the hazard(s) of a substance or mixture and

Table 1 CLP health hazard classes

CLP Annex I chapter	CLP health hazard class	Differentiation
3.1	Acute toxicity	Oral, dermal, inhalation
3.2	Skin corrosion/irritation	
3.3	Serious eye damage/eye irritation	
3.4	Respiratory or skin sensitization	Airways, skin
3.5	Germ cell mutagenicity	
3.6	Carcinogenicity	
3.7	Reproductive toxicity	Sexual function and fertility, development of the offspring
3.8	Specific target organ toxicity (STOT): single exposure	
3.9	Specific target organ toxicity (STOT): repeated exposure	
3.10	Aspiration hazard	

the need to control the associated risks. Manufacturers, importers, downstream users, or in certain cases distributors have to label the classified packaged substance or mixture before placing it on the market (Article 4 (4) of the CLP Regulation).

For the consumer, the label is usually the only source of information about the product hazards. For professional users, a Safety Data Sheet is available in addition that contains all labelling elements and substantially more hazard-related information (see section "Safety Data Sheet").

Each hazard class and category is linked to four groups of specific hazard communication elements:

- Signal word
- Hazard pictogram
- Hazard statement ("H-statements")
- Precautionary statements ("P-statements")

There are two signal words: "DANGER" with classes and categories of higher concern, strength, or potency while "WARNING" with those of lower concern, strength, or potency.

Four hazard pictograms are used in the health hazard communication: the "skull and crossbones" pictogram, the "corrosion" pictogram, the "health hazard" pictogram, and the "exclamation mark" pictogram (Fig. 1).

An overview of the links between health hazard classes/categories and signal words and hazard pictograms is presented in Fig. 2.

Hazard statements describe the character and strength of the hazard, for some classes differentiated according to the route of exposure (see Table 2 for some examples).

Precautionary statements advise about the correct handling of chemical substances and mixtures. Statements give:

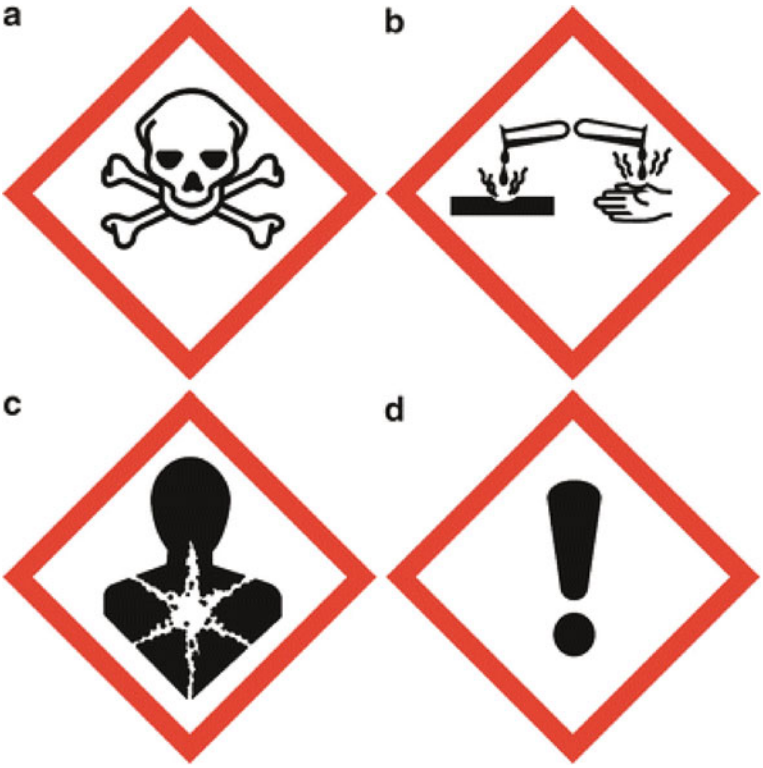

Fig. 1 Pictograms for health hazards according to the CLP Regulation (EC) No. 1272/2008 (with kind permission from UNECE 2012). (**a**) Skull and crossbones, (**b**) corrosion, (**c**) health hazard, (**d**) exclamation mark

- General advices (P1xx)
- Prevention-related advices (P2xx)
- Emergency-response-related advices (P3xx)
- Storage-related advices (P4xx)
- Disposal-related (P5xx) advices (with x indicates numeric digits)

A complete list of hazard and precautionary statements and official statement combinations (with translations in all EU languages) is included as Annex III and Annex IV to the CLP Regulation, respectively.

The system of precautionary statements is complex. For different reasons, precautionary statements are of very limited value in regulatory toxicology and therefore not further elucidated here.

All classifications of substances shall be reported to the European Chemicals Agency (ECHA). These classification and labelling information on substances are made available online in the Classification and Labelling Inventory (maintained by ECHA). This database includes all substances with a harmonized (and thus legally

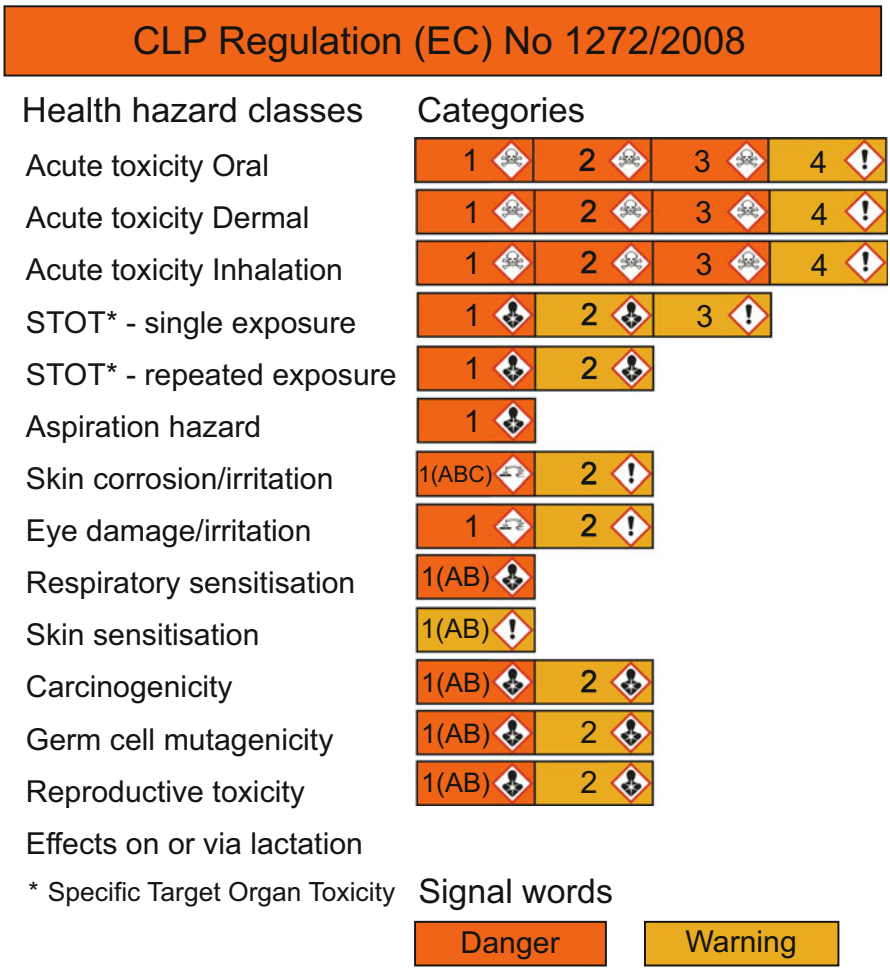

Fig. 2 Health hazard classification of substances and mixtures according to the CLP Regulation (EC) No. 1272/2008 with corresponding signal words and hazard pictograms (Adapted from Clinical Toxicology (2010) 48, 28–33 (de Groot et al. 2010))

binding) hazard classification as listed in Annex VI of the CLP Regulation and substances registered under REACH for which the manufacturer or importer is responsible for correct classification and labelling.

Safety Data Sheet

For a more detailed risk assessment, especially in emergency situations and for development of scenarios for safe use of hazardous substances and mixtures at workplace, the communication elements on the label are not sufficient. Additional

Table 2 Hazard statements for acute toxicity (selected examples for oral and dermal exposure)

Category of acute toxicity	Route	Hazard statement code	Hazard statement
1	Oral	H300	Fatal if swallowed
2	Oral	H300	Fatal if swallowed
3	Oral	H301	Toxic if swallowed
4	Oral	H302	Harmful if swallowed
1	Dermal	H310	Fatal in contact with the skin
2	Dermal	H310	Fatal in contact with the skin
3	Dermal	H311	Toxic in contact with the skin
4	Dermal	H312	Harmful in contact with the skin

Table 3 Structure of Safety Data Sheet according to REACH: sections important for emergency response

Section number	Content
1	Company name and address and emergency telephone number
2	Description of the hazards of the substance or mixture and the appropriate warning information associated with those hazards
3	Composition/information on hazardous ingredients. Listing all ingredients classified as hazardous (above specified concentration thresholds) and their concentration (either exact or ranges)
4	First aid measures by relevant routes of exposure
11	A description of the various toxicological (health) effects and the available data used to identify those effects, including, where appropriate, information on toxicokinetics, metabolism, and distribution

information is provided in the Safety Data Sheet (SDS). The SDS has a fixed structure with 16 sections, as described in Annex II of the REACH Regulation (EC) No. 1907/2006 (EC 2006). The content of the sections with important data relevant for emergency health response is listed in Table 3.

The toxicological information in Section 11 shall apply to the substance or mixture as placed on the market. If available, the relevant toxicological properties of the hazardous substances in a mixture shall also be provided. For every relevant health hazard class, toxicological information should be included, and if available, human data should be provided.

For substances, Section 11 of the SDS will include (a summary consistent with) the toxicological information which is supplied for the registration of the substance according to the REACH Regulation.

For some substances, a Chemical Safety Report (CSR) is compiled for the REACH registration which includes Exposure Scenarios describing how the substance will be used by professional users or consumers (e.g., duration and frequency) and – if needed – risk management measures to be implemented to reduce or avoid direct and indirect exposure. These exposure scenarios will be made available as an Annex to the SDS.

Poisons Centres' Perspective

As described above, consumers are informed about the hazards and safe use of a product by communication elements on the product label only, while professional users have access to additional information on the SDS. In case of incidents (unsafe exposures), the SDS information is only a starting point, and more detailed information is necessary for medical management in many cases.

When exposure cases are treated in the medical system, most often in a hospital, poisons centres are frequently consulted for professional toxicological support. Poisons centres often deal with unusual exposures, e.g., intake of large doses, untypical exposure pathways (e.g., ingestion, intravenous application), patient groups with high sensitivity to specific toxic substance (e.g., pregnant, child, immunosuppressed patients) or unusual expose pattern (patients with reduced taste, smell, or mental capacity).

An important general shortcoming of the SDS for poisons centres' practice is that only substances that are classified as hazardous have to be mentioned and only if exceeding threshold concentrations. Furthermore, as guidelines on the notification of the concentration of ingredients are not available in practice, wide concentration ranges are often used. To perform a clinical risk assessment in individual poisoning cases, poisons centres need to have access to the detailed product formula of all hazardous products.

Cross-References

- ► Assessment of Mixtures: Bridging Principles and Other Approaches
- ► Exposure Scenarios in Toxicology
- ► International Regulation of Toxicological Test Procedures
- ► National and International Collaboration in Regulatory Toxicology
- ► Notification of Hazardous Mixtures and Cosmetic Products for Poisons Centers in the European Union
- ► REACH and CLP: Its Role in Regulatory Toxicology
- ► Toxicological Risk Assessment

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Prohibition and Restrictions in Regulatory Toxicology

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Michael Schwenk and Reinhold Burr

Contents

M. Schwenk (✉)
Tübingen, Germany

Formerly: Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

R. Burr
General Practitioner Practice, Hirrlingen, Germany

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Abstract

The last century has seen huge advances in chemistry. It has been estimated that more than 300,000 substances are on the market. It is hardly possible to keep an overview of the entire complex situation. Evidently, substances that pose a risk to human health and the environment must get regulated. Even substances with low toxic risks are often restricted with regard to application fields or maximal limits.

This chapter mentions as historic example the alcohol prohibition in the United States in the 1920s. It discusses several types of prohibition and restriction, like the prohibition of chemical weapons, prohibition of certain addictive drugs, prohibition of smoking in public rooms, banning of persisting organic pollutants, and regulation of mercury. This contribution also deals with some terms used in regulatory toxicology and risk management, the praxis of prohibition, and potential disagreements and conflicts.

Keywords

Prohibition · Restriction · Ban · Alcohol · Tobacco · Biocides · Stockholm Convention · Mercury

Introduction

Considering the large number of 350,000 chemicals and mixtures that have been registered (Wang et al. 2020), each with an individual physicochemistry, reactivity, application field, life cycle, and toxicity, effective regulations and global harmonization are mandatory. Most chemicals on the market are restricted in some way, others are prohibited, usually because of toxicity to health and environment. Prohibition or banning is the most restrictive tool. They grant safety, but may affect the liberty of those who discovered, produce or use the substance.

Prohibition (from Latin prohibere = to forbid) is ordering that something not be done or used. Prohibition in regulatory toxicology is the act of banning the production, distribution, possession, or use of a chemical substance or items containing the substance. It may also be prohibited to delete a chemical from a product, where its presence guarantees quality and safety. "Prohibition" in a narrow sense refers to the period of alcohol prohibition in the USA from 1920 to 1933 (see also below).

Restriction (from Latin restrictio = limitation) in regulatory toxicology is the act of limiting and controlling the production, distribution, possession, or use of a chemical, by defining specific conditions where usage is allowed or forbidden.

Today, toxicological regulations commonly include some type of prohibition or restriction. We describe some anecdotal examples of well-known chemicals that were prohibited and restricted and the preceding, often time-consuming negotiations. Regulations may vary from country to country and change over time.

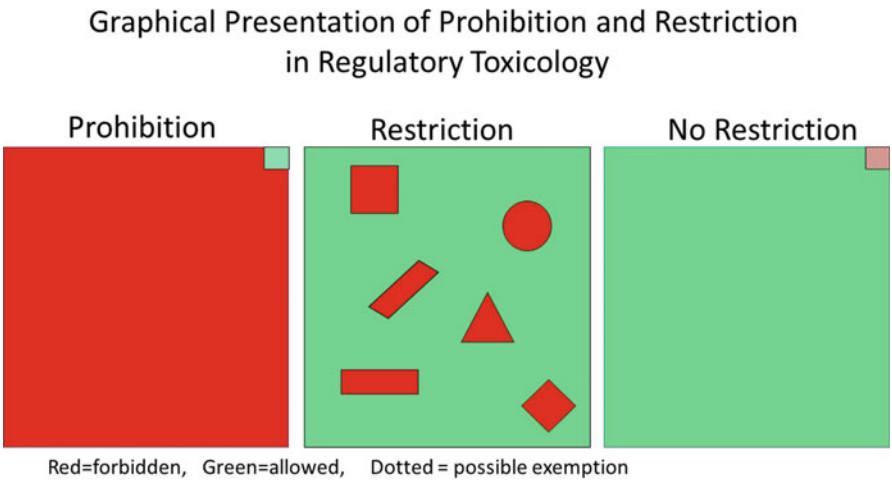

Fig. 1 Schematic visualization depicting the scopes of prohibition and restriction in regulatory toxicology

Even when a substance is prohibited, there may exist defined exemptions, allowing a specific application (Fig. 1). For example, the insecticide lindane is banned in the Stockholm Convention, but an exemption exists for (second line) medical treatment of scabies. Likewise, a substance may have a wide application, but nevertheless there may be limitations with regard to transport, ceiling levels, application fields, and others. Finally, some chemicals are believed to have no or only minimal toxicology-based regulatory limitations, like pure water. But even in such cases, there tend to exist some restrictions or recommendations (e.g., "don't spill water over metallic sodium"). The right panel of Fig. 1 may appear somewhat hypothetical.

Purpose of Prohibition in Toxicology

Prohibition in toxicological regulations can have varying targets, just to name a few:

- Prohibition concerning distribution, possession, and use of a substance outside its determined usage. This is, for example, the case for addictive drugs, some of which are on the legal market as essential pharmaceuticals when administered under the supervision of a medical doctor, but illegal for street users.
- Prohibition under any condition. Examples are chemical weapons that are prohibited in the lists of the Organization for the Prohibition of Chemical Weapons (OPCW 2021).
- Prohibition to emit or dispose hazardous substances in a way that is not approved.
- Prohibition of animal experiments, for example, in the development of cosmetics. This is incisive, considering that equivalent alternative safety-test possibilities do

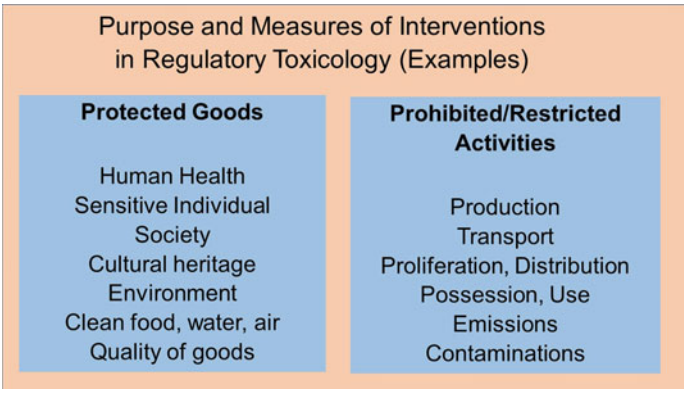

Fig. 2 Schematic presentations of protected assets and prohibited/restricted activities related to chemicals and drugs

not provide all the required information. But it reflects the intention to respect nature and life.

Examples for purpose and measures are shown in Fig. 2.

Levels and Origins of Prohibitions and Restrictions

Countries may have different governmental structures according to national laws; however, decreasing decision-making power is common when moving from national, to regional, to local regulatory authority. Prohibitions and restrictions can be proclaimed on any hierarchical level, but compliance may be better, if based on national law. An example for prohibition by local administrations is the banning of alcohol during big events in order to avoid aggressive behavior, or the interdiction to enter a danger zone after a chemical accident.

Initiatives that ultimately result in prohibition may come from scientific evidence, NGOs, whistleblowers, or court decisions. Some prohibitions are not based on secular laws, such as the prohibition to drink alcohol as a religious principle.

Decisions on the international level have the highest level of competence. International conventions are usually preceded by intensive, time-consuming international negotiations and based on scientific evidence. National interests will also be taken into account. These processes are usually very complex, resulting in an agreement of all signatory bodies. The agreements need to be adopted and incorporated into national laws.

Prohibition and restrictions both affect the freedom of individuals and industry. The problem can often get solved by replacing restricted chemicals with novel substances that fulfill the same purpose but exhibit less adversity. Chemical regulations contain prohibitions and restrictions, but also obligations, for example, to prove

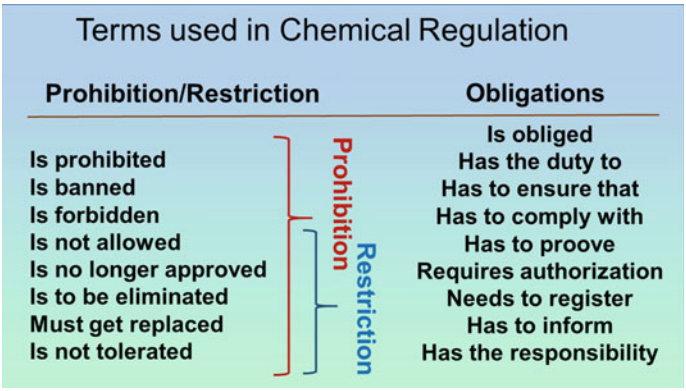

Fig. 3 Prohibitions and obligations in regulatory toxicology. The terms in the two lists are examples for expressions used by authorities. The brackets depict that there may be overlapping that may vary from case to case

the quality and safety of a product and to keep authorities informed. Figure 3 shows terms that are commonly used in regulation.

Conflicts among related regulations exist. For example, the EU cosmetic regulation bans animal testing, whereas EU REACH requires or allows animal testing of chemicals under restricted conditions to study risks to health and environment (ECHA 2017).

Drugs

Phenylbutazone was a very effective analgesic, but associated with the life-threatening side effect of agranulocytosis. In 1985, it lost its approval in Germany, meaning that a larger number of commonly used phenylbutazone-containing pharmaceutical preparations were banned from the market. In 1986, metamizol, a substance with similar pharmacological and toxic features, became restricted as well. Thereafter, medical doctors were not allowed to prescribe metamizol for common pain, but only for specific orthopedic indications, or as second line medication, when other medication was not effective. Then, over the years and decades, metamizol experienced a revival and became a major analgesic, contrary to the official recommendation. The prescribing doctors now argue that metamizol has advantages over other analgesics in having no relevant unwanted effects on blood coagulation, kidney function, and gastric mucosa and that the risk of agranulocytosis, although not exactly known, was estimated higher in the past, than it actually is. During this period, many other pharmaceuticals were restricted as well; they could usually get replaced by newer pharmaceuticals that are more effective and have less side effects.

Thalidomid came to the market in 1957. It was advertised as antinausea drug for pregnant women. It tragically produced malformations in newborns, by interfering with specific steps of morphogenesis during fetal tissue differentiation. This ended in a big

drug scandal. The drug became a prototype, showing the need for better drug testing, notably in developmental toxicology. The further use was prohibited. However, it was later detected that thalidomide has a therapeutically useful antiangiogenic effect, and the agent experienced a revival in the treatment of leprosy, as second-line therapeutic for multiple myelomas and treatment of various other diseases. It must be administered under the strictest conditions to avoid its use by women in child-bearing age. This exemplifies that new knowledge can reverse a prohibition.

Addictive Drugs

The UN "Convention Against Illicit Traffic in Narcotic Drugs and Psychotropic Substances" of 1988 by the United Nations Office on Drugs and Crime (UNODC) made it mandatory for the signatory countries to prohibit production, trafficking, sale, and distribution of certain addictive drugs that were listed in the Single Convention on Narcotic Drugs 1961 and Convention on Psychotropic Substances 1971. It also includes provisions against money laundering and related crimes.

Some current examples show the dilemma of prohibition: Carfentanyl is a synthetic opioid that is very effective as sedative and widely approved for anesthesia in large animals. It is under strict regulation. Having opioid-like psychoactive effects, it is illegally consumed by drug users. Due to its very high pharmacological/toxic potency, it is a hazardous substance in humans resulting in life-threatening inhibition of respiration.

Methamphetamine is a stimulating drug, used in some medical applications, but also misused as prohibited addictive drug. It is cheap to produce. It gets illegally pushed by the drug mafia into the international drug-user markets with a huge margin of profit. The international and national networks of prosecutors, analytical laboratories, and toxicology centers do a very good job in detecting this criminality.

This shows that the illicit drug problem has many aspects, including risks to health and socioeconomic situations of users. The authorities have difficulties to successfully eliminate these drugs and to discourage their consumption. It is difficult to find a balance between prohibition/prosecution and legalization/liberalization.

Considering the increasing influence of the drug mafia and transnational criminal activities, and the ever increasing number of novel psychoactive drug analogues sold on the black market, some people come to the conclusion that the regulations of addictive drugs according to UNODC lists (UNODC 2013) should be liberalized and decriminalized. However, it is difficult to estimate the consequences. In any case, liberalizations, such as happened with cannabis, should have several preconditions, such as:

- Mandated labels with dosage and medical warnings
- Ban on advertising
- Access to clinical setting and medical support
- Accompanying scientific control studies

Doping Drugs

Based on the world antidoping code of the year 2004 (WADA 2021) signed by the Olympic movement, national antidoping organizations and other movements, various drugs that may increase the athletic performance (e.g., stimulants, testosterone, EPO), are prohibited in sports in the annual list of substances released by the World Anti-Doping Agency (WADA). There are three categories.

1. Prohibited all times (e.g., anabolic agents)
2. Prohibited in competition (e.g., stimulants)
3. Prohibited in particular sports (beta-blockers)

Any misuse is closely controlled by WADA and its adhering national organizations; data are published. Athletes who violate these rules are publicly denounced. In general, WADA is very successful in its efforts to keep sports free of doping. It thus provides equality among athletes and breaks the spiral of performance enhancement and dose-increase of doping agents. Finally it is a necessary measure to protect the athletes from doping-induced damage to their health.

Example Alcohol

US Prohibition

Around the year 1915, various countries started to ban alcohol drinking. The best known example is that of the United States. Due to the increasing consumption of strong alcoholic beverages like whisky and rum and the expansion of breweries, alcohol consumption had increased in the preceding years. The temperance movement had the aim to ban alcohol with the arguments that alcohol disabled drinkers due to drunkenness, aggressive behavior, increasing poverty, and destruction of societal cooperation. People expected that a restriction of alcohol would concern only the liquor bars and heavy consumers. Then in 1919, the Eighteenth Amendment to the United States Constitution established that alcohol was prohibited with the following words (US law 1917):

> *After one year from the ratification of this article the manufacture, sale, or transportation of intoxicating liquors within, the importation thereof into, or the exportation thereof from the United States and all the territory subject to the jurisdiction thereof for beverage purposes is hereby prohibited*

Initially the country experienced a decline of alcohol consumption. But illegal alcohol producers soon started their work, mafia-like structures consolidated, methanol intoxication occurred, and finally the public opinion increasingly objected to the prohibition, which was finally repealed by the twenty-first amendment in 1933. Thereafter many US states issued their own regulations and restrictions. The State

of Utah is known to have preserved some strict alcohol regulations until today: Only persons older than 21 years are allowed to buy and to consume alcoholic drinks. In supermarkets, only beer with limited alcohol content is offered. Packaged liquor, wine, and beer are sold in liquor stores.

General Situation Today

It is primarily the psychoactive effects of alcohol that cause societal problems. With rising dose, alcohol induces sedation, subsequently activation, and finally – depending on personality – aggressiveness. Another risk concerns the drinking pregnant woman, because alcohol is transferred to the fetus and provides a serious dose-dependent risk to the developing fetal brain (fetal alcohol syndrome). It may have been unclear 100 years ago to which extent alcohol has chronic toxicity. Excessive chronic ethanol consumption is associated with dependency, peripheral neuropathy, brain damage, liver disorders, and increased cancer risk. When consumed by poor people in large quantities, alcohol may serve as major energy source, with the risk of malnutrition. Alcohol is associated with injuries, traffic accidents, and suicides.

Considering these adverse effects of alcohol, it may appear reasonable even today, to prohibit its use. But many people use alcohol moderately, without serious risk of dependency. And prohibition might again not be successful in many societies. According to the World Health Organization (WHO), countries have a responsibility of implementing and monitoring public policies to reduce the harmful use of alcohol and to apply evidence-based reduction measures (WHO 2018, 2021). The WHO program offers skillful tools with elaborate and evidence based indicators on reducing the negative consequences of drinking and alcohol intoxication and reducing the public health impact of illicit or informally produced alcohol. So this approach is more differentiated than a plain prohibition.

Smoking, Tobacco

In the 1970s, smoking was common in the public. Smoking was considered by smokers as a sign of independence and emancipation. Smoking was normal at the workplace, in restaurants, in airplanes even in hospitals. Few exceptions existed were smoking was not allowed, for example, near fuel stations.

Over time, individuals and NGOs who felt annoyed by cigarette smoke and restricted in their right for clean air started to organize campaigns against smoking. Initially this was not very successful, not least because the tobacco industry supported research projects related to cancer studies, with the consequence that numerous experts in the field had no interest to oppose tobacco industry; some of them were smokers themselves.

Advertisements for tobacco products continued to be obtrusive, like the depiction of a young women saying “I like smoking.” This was not tolerable, considering the

increasing knowledge about the adverse health effects on smokers, female smokers, and pregnant smokers.

A tough battle between a powerful tobacco industry and nonsmoking campaigns started. The nonsmokers argued that active smoking should be forbidden, considering tobacco is the number one toxic agent in daily use, causing atherosclerosis, lung cancer, and many other disorders. Smoking should also be forbidden because even passive smoking leads to adverse health effects in nonsmokers. In addition, many people suffer from the irritating effects of tobacco smoke on eyes and respiratory tract.

Over time, the freedom of smoking gradually became more restricted. Initially, it was prohibited to smoke at the workplace, in restaurants, and in public buildings. At the beginning of the 2000s, many countries had introduced laws that led to relevant reductions of tobacco-related risks. The debate continues, and pediatricians want to prohibit smoking in cars and homes in the presence of children.

In Europe, the Tobacco Products Directive (2014/40/EU) entered into force in 2014 and became applicable in EU countries in 2016 (EU 2021). It prohibits cigarettes and roll-your-own tobacco with characterizing flavors (that might seduce young people to start smoking because of a pleasant scent), requires health warnings on tobacco-products, allows EU countries to prohibit Internet sales of tobacco and related products, and contains many more restrictions.

Chemical Weapons

In World War I, the German army was the first to use in 1915, vaporized chlorine, a lung-toxic gas, and in addition, from 1917 on, the vesicant sulfur mustard, a corrosive agent that produces burns of the skin, irritation of airways, and lung destruction. This use of a chemical weapon was in violation of the Geneva Convention of 1864 and the Hague convention of 1899. Following World War I, the Geneva conference (1925) banned the use of all forms of chemical weapons.

In 1932, the neurotoxic action of organophosphates was detected. This led to the development of a large group of substances used as insecticides, but also to the development of very highly toxic nerve agents, such as sarin, tabun, and soman. These were produced in Germany during World War II, but not used. These nerve gases, and some newer analogues, are acutely debilitating when inhaled, often with fatal outcome or long episodes of recovery under intensive care conditions, with atropine being administered as an antidote.

The invention of these new types of agents was one of the reasons for the Chemical Weapons Convention that entered into force in 1997, headed by the Organization for the Prohibition of Chemical Weapons (OPCW) in The Hague, Netherlands (OPCW 2021). It has the mandate to stop development, production, stockpiling, transfer, and use of chemical weapons; ultimately to eliminate chemical weapons and to prevent their re-emergence. Its international inspection teams visit facilities, where chemicals are produced that might be either precursors or the final chemical weapon products. The prohibition of chemical weapons is a great step

Fig. 4 Examples for International conventions, agreements and organizations, that focus on prohibition and restriction of hazardous chemicals, chemical weapons, and/or drugs

forward towards international cooperation and humanity. OPCW received the Nobel Prize in 2013. In addition to OPCW, there exist various other international conventions and agreements that focus on prohibition (Fig. 4).

Mercury

The metals mercury, lead, and cadmium had wide applications in the past. All of them were already known in antiquity. Lead, for example, was used for lead pipes, pigments and since about the 1920s as antiknocking gasoline additive. As a consequence of human intoxications and environmental contaminations, the use of these metals was gradually restricted. Mercury will serve as example for a continuing process of restrictions and prohibitions.

Mercury sulfide (cinnabar) is the predominant mercury-containing ore, from which mercury is released by heating. Metallic mercury and most mercury compounds are very toxic, notably to the brain. However, in the past, mercury and mercury compounds had many applications.

Technical Uses

Mercury was historically used in large amounts for gold and silver extraction and until today it is used in small-scale gold mining, often under terrible hygienic conditions.

Mercury is a component of thermometers, manometers and many other measuring instruments, calomel electrodes, and in mercury batteries (widely prohibited after the 1990s). It is used as catalyst in chemical processes.

Mercuric chloride solution has been used as timber preservative (kyanizing). The soil of the treatment areas became mercury-contaminated and the sites are considered as Brownfields.

Methylmercury has fungicide activity and was used among others to preserve seeds. In 1971, a large tonnage of wheat and barley seed, treated this way, was accidently consumed in Iraq. Hundreds of people died, survivors suffered from chronic neurologic disorders. Methylmercury is toxic to the developing brain. It is transferred via the placenta to the developing fetus. This was one of the catastrophic events that later led to the ban on mercury for such applications.

Medical Uses Earlier and Now

Elemental mercury, when ingested, is poorly absorbed. Elemental mercury was previously used to treat acute intestinal obstruction. Mercury was also the main component of "beauty cream" used to lighten the skin and is still found in the markets of some regions in the world (FDA 2016).

Mercury(I)chloride (Hg_2Cl_2, calomel) is poorly soluble and less toxic. It was used as laxative and diuretic.

Mercury(II)chloride $HgCl_2$ (corrosive sublimate) is water soluble, tends to sublime, and is very toxic. It has been in use as antiseptic and to treat syphilis for 500 years. The mercury therapy often led to chronic inhalative mercury poisoning. It was abandoned, when the first effective antimicrobial agent, salvarsan, became available in 1910.

Mersalyl is an organomercury compound that was formerly used as diuretic as it acts on renal tubules and decreases blood pressure and edema. Today it is still used in biochemistry as an agent that protects sulfhydryl groups in proteins.

Merbromin, a mercury- and bromine containing red-colored organic substance, was used in aqueous solution as powerful disinfectant for wounds and burns. When analytical methods became available for measuring mercury in blood, it was noticed that merbromin treatment increases mercury levels in the blood. This was a major reason for the US FDA to declare it not safe for general use in 1998. It was thereafter withdrawn from the markets in several countries, partly without the need for explicit prohibition, because the producers were not able to proof the safety in comparison to alternative disinfectants.

Thiomersal, an organochlorine compound, is used in small concentrations as a preservative in vaccines. Near the end of the last century, there was some concern that it might induce autism. However, WHO came to the following conclusion: "Based on the current evidence, GACVS considers that no additional studies of the safety of thiomersal in vaccines are warranted and that available evidence strongly supports the safety of the use of thiomersal as a preservative for inactivated vaccines" (WHO 2012).

Mercury amalgam tooth fillings were state of the art until the 1990s. They have the advantage of not requiring sophisticated technical equipment and being cheap for people who cannot afford more elaborate fillings. Tooth amalgam is very insoluble, but corrodes over time and releases tiny amalgam particles that may be swallowed. Because of their low solubility and rapid passage through the gastrointestinal tract, these are believed not to contribute in relevant amounts to the internal load. Nonetheless, people with amalgam fillings tend to have elevated mercury levels in the blood, dependent on the amount, quality, and age of the fillings. This is thought to occur via the inhalative pathway. This finding, together with the risk of exposure for dentists and their coworkers and the problem of environmental contamination, led to the initiation of campaigns against dental amalgam, which resulted in restrictions. These may differ between countries. A recommendation in the Minamata convention suggests to prefer mercury-free dental restauration (UNEP 2017).

Mercury and the Environment

Metallic mercury that is spilled indoors and not removed will evaporate and result in long-lasting elevated indoor air concentrations, a risk for those who live or work in these rooms.

Environmental mercury in soil and water is gradually converted by plankton and microorganism to methylmercury. This is a very lipophilic substance that accumulates in the nutrition chain, typically in fish of mercury-contaminated waters, ultimately constituting a relevant source of mercury for fish-consuming humans. Some countries have developed recommendations for pregnant women, to reduce consumption of larger fatty fish, in order to minimized exposure to methylmercury (and POP).

Mercury Regulations

As outlined, mercury had widespread applications until the middle of the last century when evidence for adverse mercury contaminations and mercury levels in the human body and the environment could be analyzed with increasing precision. Decades followed when mercury containing products were prohibited or restricted step by step. Mercury may serve as an example for multiple regulations (restriction and prohibition) on national and international levels. For example, the EU set maximum levels for mercury and many other contaminants in foods (EU 2006). The Minamata Convention on mercury provides insight into the dimensions of the mercury problem and its suggested regulations (UNEP 2017). The convention is a global treaty to protect human health and the environment. It is named after the mercury incidents in Minamata Bay, Japan. It was adopted in 2013 and entered into force in 2017. It includes a ban on new mercury mines, the phasing-out of existing mines, and reduction of mercury-containing products, processes, and waste, as well as surveillance of emissions and exposures.

The Global Mercury Assessment (UNEP 2018) is a collection of worldwide data on mercury emissions and exposures. It provides a departing point, which will allow to estimate the effects of prohibitions and restrictions in the future.

Persistent Organic Pollutants (POP)

The Stockholm Convention (UNEP 2004) is a global treaty to protect human health and the environment from persistent organic pollutants (POPs). POPs are chemicals that remain intact in the environment for long periods, become widely distributed geographically, accumulate in the fatty tissue of living organisms, and are toxic to humans and wildlife. POPs circulate globally and can cause damage wherever they travel. In implementing the convention, governments will take measures to eliminate or reduce the release of POPs into the environment. Over 152 countries ratified the Convention and it entered into force in 2004.

The Stockholm Convention has elements of prohibition and of restriction for each of the persistent organic pollutants (POPs), listed in the Annexes of the convention. DDT, PCBs, and PCDDs are members of the original list of 12 regulated substances. Many other organochlorine substances and nonchlorinated substances are currently on the list (UNIDO 2021).

We take here lindane (hexachlorocyclohexane) as an example. Lindane was formerly widely used in many countries as insecticide to eliminate mosquitos and bugs. Production and agricultural use is now banned in the countries that are Parties to the Stockholm Convention. However, lindane may be used as second-line medical treatment for ectoparasites on and in the human skin (lice and scabies) when other agents are ineffective or not tolerated. In most countries, today, the first choice insecticide for this application is permethrin. Lindane should be used with caution, notably in children, because of absorption via the skin, accumulation in tissues, long half-life, and neurotoxicity of the agent.

DDT (dichlordiphenyltrichlorethane) was synthesized already in the nineteenth century. Its insecticidal action was discovered in 1939 by the Swiss chemist Müller, who was awarded the Nobel prize for discovering this very efficient contact poison against insects. DDT came to market in 1942 and was rapidly found to eliminate malaria by killing the anopheles mosquito.

DDT is a chlorinated organic compound that is very lipophilic and resistant to degradation in the environment and indoors, meaning that it persists for a long time, once sprayed on surfaces. This is an application-advantage. In humans, it is subject to metabolic transformation to DDE and DDD, which then both accumulate in the body fat and in milk fat and are thus transferred to babies. DDT also induces cytochrome P450 and is a tumor promotor.

Already in 1962, Rachel Carson wrote in her book “Silent Spring” about the dangers of chemical pollution. She argued that the number of fish eating birds decreased, because DDT accumulated in fish through the food chain which seemed to make the eggshells thinner and more fragile. Carson wrote just a booklet not a high-level publication. Nevertheless, her argumentation received much attention

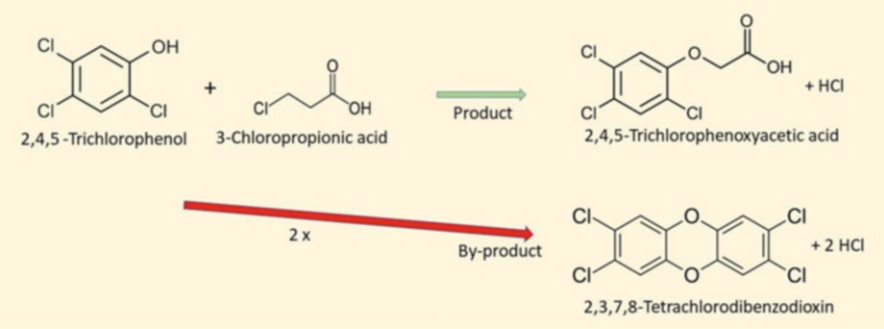

Fig. 5 Formation of the by-product 2,3,7,8 tetrachlorodibenzodioxine. The herbicide 2,4,5-trichlorophenoxyacetic acid is synthesized in a reaction of 2,4,5-trichlorophenol with 3-chloro propionic acid, where two molecules of 2,4,5-trichlorophenoxyacetic may combine to the minor by-product 2,3,7,8-tetrachloro-dibenzodioxine, which occurred as a contaminant of the herbicide

among environmentalists at the time, and the DDT-dilemma resulted in a ban of DDT for agricultural uses in the United States in 1972 and was one of the arguments that led to bans in many other countries.

However, at the same time, DDT was still considered an important agent for fighting insect-born disease in various countries. Although basically banned in the Stockholm Convention, it received an exemption for disease vector control in specific circumstances. Meanwhile there is some evidence that the thinning of eggshells may be due to the hormonal effect of DDT.

TCDD is not an industrial chemical. It has never been produced for an application, but became known as an unintentional by-product of certain chemical reactions. It occurred as contamination in the defoliation agent "agent orange," a mixture having 2,4,5-T and 2,4-D as active herbicides (Fig. 5) that was used in the Vietnam war and resulted in TCDD contaminations of plants, fields, domestic animals, adults, and babies (via mother milk). TCDD is also a very common side product of incineration processes when chlorine-containing organic material is heated in the presence of oxygen. TCDD is the most toxic and best known congener in the family of polychlorinated dibenzodioxines.

Biocides

The group of insecticides includes chlorinated organic compounds, organophosphates, carbamates, pyrethroids, and neonicotinoids. All of them are neurotoxic to insects and to humans. Many members of these biocides have been prohibited for different reasons, including residues on edible crops, persistence in the environment, contamination of ground water, unintended extinction of useful insects like honey bees, as well as risks of acute neurotoxicity to workers and elevated risks of suicidal application.

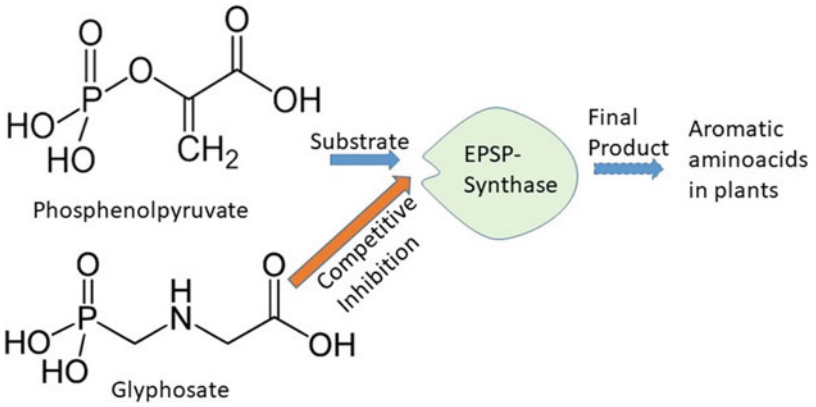

Fig. 6 Structures of the natural substrate phosphoenolpyruvate of the plant-enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase and of the competitive inhibitor glyphosate. EPSP synthase has as a second substrate shikimate-3-phosphate (not shown). The reaction product is a precursor of essential aromatic amino acids in plants

Neonicotinoids are a comparatively new group of insecticides. They are very effective in insects. There was an extensive discussion about their negative effects on honey bee colonies. Neonicotinoids are widely banned in the European Union from 2020 on, but there are exemptions for certain applications (EU 2020b).

Another group of biocides are the herbicides, intended to suppress growth of weed. One of the most controversial biocides is glyphosate. It was introduced in 1975 as herbicide that is specifically toxic to plants, bacteria, and fungi, but not to animals. Glyphosate inhibits competitively the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which does not occur in animals, but is member of an essential biochemical pathway in bacteria, fungi, and plants (Fig. 6). Its inhibition leads to a deficiency of aromatic amino acids, and thus, to death of plants. Glyphosate is widely used in agriculture to combat weeds that compete with cultivated crops, but also for other reasons like keeping railway tracks free from overgrowth. Glyphosate is usually applied before crops are sown and/or in combination with genetically modified, glyphosate-resistant crops. Glyphosate minimizes the need to use ploughing machines.

The International Agency for Research on Cancer (IARC) classified glyphosate as “probably carcinogenic to humans” in 2015. Thereafter, a never ending dispute between those, supporting this classification, on the basis that very high exposures of humans has been found to be associated with multiple myeloma and others, who found no evidence for carcinogenicity (Andreotti et al. 2018). This situation led to diverse regulatory responses in different countries. The situation for regulatory agencies is complex because, first, the risk assessment results of IARC are usually not put in question, and second, because some see a major risk of glyphosate in the concomitant use of genetically altered crops and a risk for the future of global agriculture. They oppose the use of genetically resistant crops and anticipate damage

to biodiversity (no wild plants = no bees and insects = no animals that would feed on insects. . ..) and contamination of soils and ground water.

Glyphosate is currently approved in the European Union until December 2022. It can be used by member states until this date, subject to each plant protection product being authorized by national authorities following an evaluation of its safety. Thereafter, it is intended to initiate a renewal process for future approval of glyphosate in the European Union (EU 2020a). Germany intents to prohibit glyphosate from end of 2023 (BMU 2021).

Food, Drinking Water

Food is a potential source for chronical intake of toxic substances. These can occur as natural ingredients of a food (lectins in beans), contaminants (aflatoxin on poorly stored rice), pesticide residues, food additives, or intentionally added substances, used to adulterate a food (e.g., melamine addition to milk in order to imitate a high protein level). Numerous prohibitions and restrictions exist, often defining maximum levels (EU 2006, 2011). A network of controlling agencies with well-equipped analytical laboratories was established in many countries which are specialized in detecting substances that are prohibited or regulated in foodstuff.

An example for the regulation of natural plant products is the pyrrolizidine alkaloids that occur in many plants and to which humans may be exposed via certain teas and leaves. When ingested, they are metabolized via reactive intermediates that are genotoxic and hepatotoxic. Maximal levels of pyrrolizidine alkaloids were defined in various foodstuffs, notably tea and some herbal products (EU 2020c).

Similarly, drinking water may become contaminated in various ways, including geogenic substances (e.g., arsenic), contaminants (such as nitrate from agricultural activity), residues (from plant protection agents), disinfection side products (from reactions of chlorine with organic substances in the raw water), or released materials from water pipes. The drinking water guidelines of the World Health Organization (WHO) provide toxicologically derived limit values (WHO 2017). In addition, technological aspects require consideration. Although drinking water is a relevant source for calcium in humans, it is common to limit the calcium content of drinking water in order to prevent calcification of pipes and washing machines.

Regulations of Emissions and Waste

Since the Great Smog of London (1952), there were continuous technical improvements to reduce harmful emissions from industrial activities, homes, and automobiles based on effective filtering techniques. Regulations attempt to limit emissions and thus keep the outdoor air quality within reasonable levels. The standards differ from country to country. More recently, a focus of regulation has been nitrogen oxide emissions from diesel engines, ultrafine particles, photo-smog, and volatile organic substances.

Unresolved is the problem of carbon dioxide emissions. Carbon dioxide is not toxic at the still low atmospheric levels, but emissions contribute to the elevation of atmospheric temperature. Carbon dioxide is considered to be the most relevant anthropogenic factor that influences climate. The Paris agreement on climate change aims at reducing carbon dioxide emissions to an extent that would not result in further warming of the atmosphere (UN 2016). The concepts aim at replacing natural oil, gas, and coal by other energy producing technologies that do not emit carbon dioxide. However, this is a big challenge that will require lots of innovations in climate-friendly energy production with global cooperation.

Hazardous waste, environmental plastic, and electronic waste provide an ever increasing problem, notably in the high-throughput, throw-away societies that accumulate endless masses of waste. The Basel convention on transboundary hazardous waste control (UNEP 2019) attempts to find ways out of some of these dilemmas.

Nuclear Weapons

The treaty on the prohibition of nuclear weapons entered into force in 2021 (UNODA 2021). The treaty aims at sustainability and prevention of nuclear wars. Though physical aspects are in the focus, it also is of radio-toxicological relevance, considering possible exposures during isotope enrichment and separation, or, in the case of an incident, the acute- and chronic exposures to radio nucleotides by inhalation (iodine) or ingestion (cesium). By 2021, those countries that possess nuclear weapons and some of their partners did not sign the convention. Nevertheless, it is the hope that the convention will develop into a general agreement that results in the step-wise elimination of nuclear weapons.

REACH and Restrictions

REACH is a regulation of the European Union that stands for Registration, Evaluation, Authorization and Restriction of Chemicals (ECHA 2006). It entered into force in 2007. It is very voluminous and considered one of the stricter regulations.

REACH has the aim to protect human health and the environment from risks, posed by chemicals. It also aims at reducing tests in animals. The most hazardous substances should be substituted with priority by less dangerous ones. The standard process is via registration. Substances of very high concern (notably carcinogenic, mutagenic, reprotoxic) may require an authorization in order to be placed on the market (ECHA 2021).

The British government has issued in connection with the EU exit, the domestic UK-REACH Regulations (UK 2020), which are largely congruent with EU REACH (ECHA 2006), with changes to make it operable in a domestic context. The key principles of the EU REACH Regulation have been retained. From 2021 on, the EU REACH and UK REACH work independently from each other. This demonstrates that regulations are in a continuous, dynamic flow.

Summary

A huge number of laws and conventions aimed to prohibit chemicals and drugs have been established since the 1980s, many of them after 2000. The word prohibition may sound authoritarian to some, and related terms are often used such as "banned," "not allowed," "forbidden." The setting of rules for chemicals is extremely complex. It restricts essential interests, such as protection of property, freedom of trade, and freedom of individuals, in the interest of human health and preservation of the global environment. Toxicology as a basic and applied science is of imminent importance for the management of these processes. With the definition of limit values, toxicology provides the basis for safe and acceptable solutions. These processes have to continue, considering the upcoming new challenges, like biopharmaceuticals, nanoparticulate matter, waste accumulation, and global pollution.

Cross-References

▶ Chemical and Biological Weapons and Their Regulation
▶ Limit Value Setting in Different Regulatory Areas of Toxicology
▶ Precaution Principle Versus Danger Prevention in Toxicology
▶ Protected Property and Protection Level in Regulatory Toxicology
▶ Psychoactive Designer Drugs: Classes, Mechanisms, and Regulation
▶ REACH and CLP: Its Role in Regulatory Toxicology
▶ Regulation of Agrochemicals
▶ The Regulatory Process in Toxicology
▶ Tobacco and Alternative Nicotine Products and Their Regulation

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Susceptible Population Groups in Regulatory Toxicology

70

Ursula Gundert-Remy

Contents

Abstract

Awareness has increased that subgroups in the population may exist which respond with a higher susceptibility than the majority of the population when setting health-based threshold values or assessing MOS (margin of safety) values. It is not an easy task to take increased susceptibility into consideration. Even if we know or at least have some indications that, for example, new-borns might be more susceptible than adults, it is hard to account for the difference in terms of numeric figures because of incomplete data.

U. Gundert-Remy (✉)
Institute for Clinical Pharmacology and Toxicology/Institut für Klinische Pharmakologie und Toxikologie, Charité Universitätsmedizin Berlin, Berlin, Germany
e-mail: ursula.gundert-remy@charite.de

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Keywords

Specific subgroups in the population · Neonates · Elderly · Unborn child

Introduction

When assessing substances or exposure situations, for example, accidental release of dangerous chemicals, it has to be considered whether in the remit of the assessment, susceptible subpopulations may exist. If so their specific situation has to be reflected in the assessment and the measures taken. Potential sensible subgroups are the unborn child, the neonates, the elderly, and the subjects with allergic conditions.

Are Susceptible Subgroups Protected by the Default Safety Factors?

Intraspecies variability and susceptibility has to be taken into consideration when setting health-based threshold levels like ADI (accepted daily intake) values for pesticides. The conventional default value of 10 has been introduced by JECFA (Joint Food and Agriculture Organization – World Health Organization Expert Committee on Food Additives) in the 1950s and was taken up by Joint Food and Agriculture Organization – World Health Organization Meetings on Pesticide Residues (JMPR). The default factor of 10 was subdivided in a factor covering variability in toxicokinetics and a subfactor covering variability in toxicodynamics. It has been proposed to allocate equal factors of 3.14 for both parts of the total factors. Other scientists proposed to use a subfactor of 4 for the toxicokinetic and a subfactor of 2.5 to cover variability in toxicodynamics. Retrospective analysis of data from clinical studies shows that the factor of 10 is sufficient to cover intraindividual variability with the exception of cases in which the drug is metabolized by a polymorphically expressed enzyme (e.g., CYP 2D6 or CYP 2C18). As the data were mainly from phase-I studies in which health young adults with predefined body weight and body height take part, the variability of the general population may be greater than in the population of young healthy adults.

Specific Groups and Regulations

The need to consider special subpopulations is laid down in several guidances for risk assessment and for the use of the TTC (threshold of toxicological concern) concept (EFSA et al. 2012, 2019). However, there is no general factor to be used and a case-by-case consideration is recommended.

When deriving the ADI, consideration is given to population groups above 16 weeks of age only. It is only recently that a specific risk assessment is performed for infants below 16 weeks concerning food additives which are allowed to be used in formulas such as modified starch.

Table 1 Examples for specific regulation for children

Regulation	Specific parameter
Ordinance on toys for children	Heavy metals
Order on dietetic foodstuff	Organochloro- compounds
Protection of minors	Alcohol
Drug law	Specific doses for children, contraindications

In Germany, the protection of children is explicitly expressed in several regulations. Table 1 gives some examples.

A second group which is often mentioned as being specifically sensitive is the group of pregnant women. It should be born in mind that it is not the pregnant women who is the sensitive subject but the unborn child whose development when exposed to environmental may be impaired.

Other groups are the elderly which are mentioned when discussing about specific sensitivity towards exposure against chemicals, the female population in general, and asthmatics. The drug legislation foresees specific trials for children. Elderly and women have to be included into the trial population and their data may be requested to be analyzed separately. In the following part, we will discuss the state of the knowledge including also subjects with impaired excretory organ function.

Specific Sensitivity in Different Life Periods

Prenatal Period

Birth defects due to prenatal exposure towards chemical substances, drugs, infections, and other environmental influences are the main causes for mortality in early life. Drugs which are known to cause birth defects are among the groups of cytostatic drugs, sex hormones, anticonvulsives (such as valproic acid), and antipsychotics. Intrauterine infections which can cause birth defects are rubella, cytomegalovirus infections, syphilis, and toxoplasmosis. Radiation at higher doses may cause structural abnormalities of the brain and the eye. Exposure against some chemicals and environmental agents during pregnancy has been described as causing birth defects. Organic solvent sniffing may cause craniofacial abnormalities, similar to the syndrome after alcohol abuse in pregnancy. Further defects are associated with glycol ethers and alcohol abuse such as microcephalus and intellectual impairment. Some other agents have been imputed. The data are however inconclusive including pesticides exposure and arsine exposure. Also, the exposure of the father has been imputed to be causally related to birth defects without clear results (tobacco, grass, pesticides, anesthetics, and lead).

It is however beyond doubt that smoking, even secondary smoke during pregnancy, is related with low birth weight.

The influence of intrauterine exposure towards chemicals on the development of cancer is controversial in most of the cases. Diethylstilbestrol is known as causing vaginal carcinoma in young adults, 20 years after intrauterine exposure.

Age up to Six Months

In this period starting at birth, toxicokinetic differences are well documented. The activity of most of the CYP enzymes is lower at birth, maturating during the first 6 months and reaching the level of the adult within the first year. The enzyme proteins for phase-II conjugation reactions are expressed whereby for some glucuronyl transferases, the levels at birth are at the adult level, for others a reduced capacity is known (e.g., UTG 1A1 or UTG1B15). As far as known today, sulfation is fully expressed at birth, whereas activity of acetyltransferase may reach adult levels only at the age of 2. Absorption through skin is enhanced due to the reduced thickness. Distribution of substances is different because of the lower relation between fat and body weight and the higher relation between water and body weight. In the first weeks, the blood/brain barrier is not anatomically developed which plays a role in the development of "kernicterus" in the newborn where physiologically a high turnover of erythrocytes containing the fetal hemoglobin in newborns leads to high bilirubin levels. In cases of different blood groups between mother and child, the incompatibility leads to lysis of erythrocytes. The high bilirubin levels in conjunction with the impaired ability to conjugate bilirubin to bilirubin glucuronide and the impaired blood/brain barrier are components of the enrichment of bilirubin in cerebral structures such as basal ganglia and brainstem nuclei. The status "kernicterus" may lead to severe neurological deficit and even death.

The renal function as measured by glomerular filtration rate is reduced up to 50% in early life and is gradually increasing to the normal level within the first 6 months. The physiological changes are all in the direction that the elimination and excretion of xenobiotics is prolonged which at the same level of exposure leads to higher internal exposure when compared with the adult. Depending on whether the parent compound or the metabolite is the toxic agent, this may result in a high (parent compound is the toxic agent) or lower (metabolite is the toxic agent) sensitivity. Brain, bones, immune system, and endocrine and reproductive organs are developing in the postnatal period over years. Thus, specific susceptibility can be present also after the first months in life. In particular the development of the brain is a highly susceptible physiological process which might be critical. In this respect, lead and also PCBs may play a role in negatively influencing intellectual capacity. The specificities of this age group and how to consider them in the risk assessment are explained in detail in the EFSA Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age (EFSA SC et al. 2017).

Child and Youth

The kinetics in these age groups is not much different from the kinetics in the adults. In school-aged kids, the metabolizing enzymes are rather highly expressed so that the clearance is somewhat higher than in adults. It is to be noted that windows of development exist during which exposure towards chemicals may have negative influences. Continuously developing organs are the brain (influence of marihuana?) and bones. Closure of epiphyses can be influenced by chinolones. The immune system is developing until the age of young adults. Exposure towards some chemicals might negatively influence the development of the immune system. Among the chemicals imputed to cause immunotoxicity are organotin compounds, pesticides (methoxychlor, heptachlor), and polyhalogenated aromatic hydrocarbons (e.g., 2,3,7,8-tetrachlordibenzo-p-dioxine). A special awareness has been raised for the exposure towards compounds exhibiting sexual hormonal activity, so-called endocrine active substances. There is no doubt that high exposure levels can influence the sexual development in animals; the effect of much lower exposure levels in the human population is not yet finally assessed.

Elderly (>70 Years of Life)

Physiological functions may be caning with increasing age whereby the variability of these processes is extremely high. Generally, the excretory functions are declining with age. Liver mass as well as liver blood flow are slightly reduced in high age (mass, reduction of 17% between the age of 20 and the age of 80 years; liver blood flow, reduction of 0.3–1.5%/year). Reduced liver mass and liver blood flow may cause a reduced metabolic clearance of chemical compounds. Depending on whether the parent compound or the metabolite is the toxic agent, this may result in a high (parent compound is the toxic agent) or lower (metabolite is the toxic agent) sensitivity. The renal function measured by glomerular filtration rate is also a function of age. The physiological change is a reduced renal blood flow (2%/year) and a reduced renal organ mass (up to 30% in the very elderly). Substances which are excreted by the kidneys (e.g., perfluorooctanoic acid, PFOA) are much slower excreted. It should be noted that at the same external exposure level, a twofold higher internal exposure will result if the renal function is reduced to 50%.

On the toxicodynamic field, there are some observations showing a reduced number of receptors. This concerns beta-receptors, alpha-2 receptors, and insulin-, glucagon-, steroid-, dopamine-, and prolactin-receptors. The results, if transferred to the in vivo situation, should result in a lower susceptibility. Whereas for some effects (effect of beta-blockers, effect of insulin), data in humans and animals are available, no generalization can be made, and for chemicals this field is not yet studied in detail.

Pregnancy

It is well known that the body of the pregnant woman undergoes physiological changes with an increased blood volume, body weight, and hormonal changes. Nevertheless, the difference between pregnant women and nonpregnant women for three substances (caffeine, midazolam, and metoprolol) is not more than a factor of two. Hence, major and important changes are not present.

Influences of Diseases

Impairment of Excretory Organs

Impairments of excretory function in liver or renal diseases are the same as described for the very elderly. At the same level of external exposure, the level of internal exposure is several times higher as compared with healthy subjects, potentially causing an increased effect. Theoretically, in patients with reduced lung surface such as in emphysema patients, the reduced surface should lead to a reduced internal exposure. On the other hand, substances which are excreted by exhalation should accumulate in these patients. However, there are no data confirming the theoretical considerations. It is known that in emphysema patients and in patients with asthma, chemicals acting on the airways have an increased effect ("higher sensibility" of these patients).

Other Organs

Systematic studies are not found in the literature. It is to be inferred from biology that patients with impaired bone marrow due to pretreatment with cytostatic drugs may have an increased effect from chemicals acting on the blood and bone marrow such as benzene. Likewise, patients with impaired immune function either inborn or due to treatment with immunosuppressive drugs may be at a greater risk than healthy subjects exposed towards immunotoxicants. In the current risk assessment, no special considerations apply for subgroups with preexisting conditions.

Gender-Specific Aspects

Physiological differences are obvious between males and females. Different chromosomal status and hormonal levels determine the phenotypic body appearance, in particular in the adult life. Men do have higher mean body weights and higher mean height when compared with the mean values for women. The body composition differs in such that men have a greater muscle mass in terms of proportion of their body weight, whereas women do have a greater proportion as fat mass.

The hormonal situation in women is characterized by cyclic changes in the estrogens and gestagens in the lifetime between menarche until menopause; in a great number of women using hormonal contraception, the situation is changed. During pregnancy the level of hormones increases from about 50 pg/ml (ovulation) to 25 ng/ml in week 40 of pregnancy; this is a factor of 500. In the menopause, the sex hormonal levels are declining. In males extreme changes in sex hormonal levels are not known.

Gender-related differences of metabolizing enzymes are known; they are, however, not impressive. The activity of CYP3A4 is some 20% higher in women as compared to men. There are subtle changes of hormonal influences on CYP 1A2 activity as the half-life of theophylline and caffeine may be different within the cycle, and men do have a bit higher activity of CYP1A2. As the changes and differences are rather small, they are covered in the intraspecies factor of 10.

In animal studies, we know some sex-specific reaction and the results of studies have to be assessed cautiously. Renal tubular damage related to α2-microglobulin excretion is found in male rats only, and the finding is without relevance for the human, males, as well as females. On the other hand, breast tumors in female rats are difficult to assess with respect to the relevance for humans as it is the leading tumor in females. It may also occur in men, however, only in rare cases. Carcinoma of the prostate and mesothelioma of the testes are clearly only occurring in males. Thus, findings in animals are difficult to interpret.

Although physiological differences and sex differences in susceptibility and findings in animals exist, we do not know similar differences towards chemicals, with the notable difference of sex hormones. At present, the effect of endocrine active substances is hotly debated. The question remains unresolved until now whether these substances in environmental concentration may negatively impact male fertility. Also the increase in breast cancer in females has been discussed in the context of exposure towards chemicals with estrogenic activity present in the environment.

Recently, gender-specific aspects are taken up in developing drugs and treatment of diseases (Legato 2017).

Cross-References

► Benchmark Dose Approach in Regulatory Toxicology
► Toxicological Risk Assessment

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Toxicology Report 71

Thomas Gudermann, Andreas Breit, Ingrid Boekhoff, John H. Duffus, H. M. Bolt, and Harald Mückter

Contents

T. Gudermann (✉) · A. Breit · I. Boekhoff · H. Mückter
Walther Straub Institute of Pharmacology and Toxicology, Ludwig-Maximilians-Universität München, Munich, Germany
e-mail: thomas.gudermann@lrz.uni-muenchen.de; andreas.breit@lrz.uni-muenchen.de; ingrid.boekhoff@lrz.uni-muenchen.de

J. H. Duffus
Toxicology, The Edinburgh Centre of Toxicology, Edinburgh, Scotland, UK

H. M. Bolt
Leibniz Research Centre for Working Environment and Human Factors, Dortmund, Germany
e-mail: bolt@ifado.de

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Abstract

The question of whether or not a substance should be classified as toxic could in certain cases be a question of life or death. Despite or because of this importance, this critical question is surprisingly hard to answer, since many different aspects, which may influence the toxicity of a compound, have to be considered. In addition, scientist or authorities with a differential expert knowledge and years of experience might differently interpret registered compound-related effects. Thus, writing of an unbiased toxicology report might be the best way of evaluating the toxicological significance of a given compound. Such a report should aim at presenting all collected raw data, the way of calculating the statistical significance, an evaluation of the potential health risks associated with the exposure to a defined compound, and the complete related literature. Then, in the best case, such a toxicological report answers the question about a possible toxic effect of a given substance or at least is the basics of further analysis or investigations. Although every toxicological report is unique and requires adjustment to the predefined study design, one has to keep in mind that such a report in some cases represents the base of legislation or in the worst case can make the difference between life and death. In this review, we give an overview of scientific, industrial, and consumer-related toxicological reports; summarize essential knowledge and key changes regarding exposure, poisons, and poisoning; and indicate contents, which are required to evaluate the toxicity of a tested compound.

Keywords

Toxicological report · Toxicological Analysis Report · Toxicological Assessment Report · Toxicological endpoints · Contergan Trial

Key Points

Toxicologists may be asked for their expertise in academic, criminal, labor, social, and civil affairs.

Typical starting points for expert reports are private or public service remits, sometimes also a court direction for evidence, which is mandatory and specific to the expert.

An expert report has to meet typical requirements in form and content as outlined below.

What's New?

Since the release of the first edition, several changes have gained impact on how we discuss, communicate, and report toxicology-related findings and conclusions. However, in spite of almost a decade of scientific advance, there is still no generally

consented standard on what should be included in the Toxicology Report and how it should be presented. Some organizations have implemented their own layouts, and we will present a few of them.

This chapter summarizes essential knowledge and key changes regarding scientific, industrial, and consumer-related reports on exposure, poisons, and poisoning. The revision of several regulations dealing with classified and public information has reshaped the typical way to write and comment on toxicological findings and health hazards. A great deal of toxicological expertise is required in court. However, this extensive field of forensic toxicology is mostly beyond the scope of this chapter, and the inclined reader is referred to special publications outlined in the "Further Reading" section.

Expert Requirements

A toxicologist is a respected expert. Advanced education is required, and several years of professional expertise is the typical outset to become a toxicologist. Former training programs saw many adepts emerging from medical specialties like pharmacology, occupational health, and hygiene, but a web article by Jennifer Alyson (https://work.chron.com/duties-toxicologist-16885.html) states that a bachelor's degree in biology, biochemistry, chemistry, or environmental science currently characterizes the majority of applicants. Not surprisingly, according to a recent publication of the Society of Toxicology (SOT), the world's leading group of toxicologists, most graduates specializing in toxicology find their jobs in industry, academia, and agencies that are government-related or work for nonprofit organizations dealing with environmental health or workplace and consumer safety (Fig. 1).

Toxicologists working in various fields after graduation often join one or more of the major societies, associations, and networks worldwide, where they find early information on emerging issues and support to organize their Reports according to specific needs. Table 1 lists the major associations (and their websites) where experts gather to communicate, discuss, and elaborate on the latest toxicology challenges and controversies. These communities are also best places where to get the most recent information about report requirements, standard layouts, and citation conventions. Apart from a deep understanding of the scientific foundations, a fundamental knowledge of the specific field of law is another important expert requirement. The expert should approach the subject and its related terms from the point of view of a lawyer, in order to be able to explain any investigations, methods, and results and communicate these findings in court. The dictionary of toxicological terms has been growing rapidly over the past 40 years, and even experts sometimes miss the mark when a concise distinction of terms is necessary. Another requirement is clear and unambiguous presentation – in written and oral form – of the details and facts pertaining to the case. Expert witnesses may be challenged by critical questions posed by the scientific community and legal authorities. Outside of academia, it is not the expert's primary goal to come up with new hypotheses or to forward suspicions outside the realm of toxicology but to communicate the corroborated

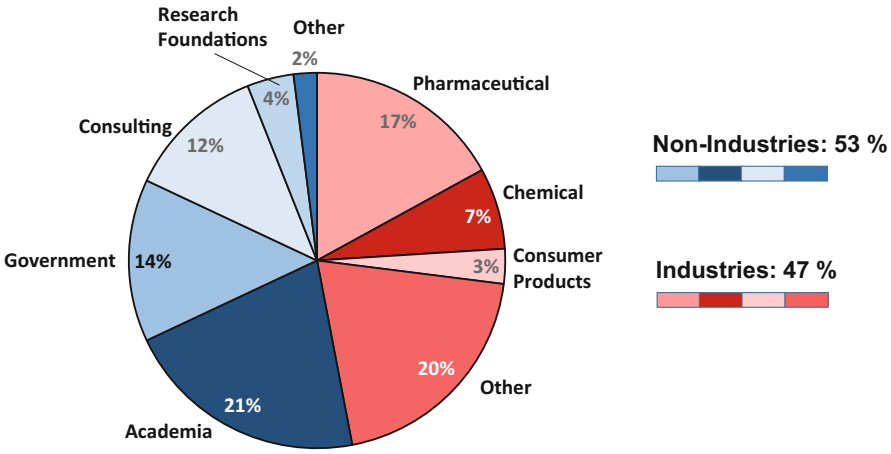

Fig. 1 Employment of toxicologists after graduation. (Modified from: 6th Global Summit on Toxicology and Applied Pharmacology 2016, available from: https://toxicology.global-summit.com/2016/)

Table 1 Selected toxicology societies and their websites

Society	Homepage
Society of Toxicology, USA (SOT)	https://www.toxicology.org/
Society of Toxicology of Canada	https://stcweb.ca/
EUROTOX	http://www.eurotox.com/
British Toxicology Society (BTS)	https://www.thebts.org/
French Society of Toxicology (STF)	https://www.sftox.com/
German Society of Toxicology (GT)	https://www.toxikologie.de/
Italian Society of Toxicology (SITOX)	https://www.sitox.org/
Japanese Society of Toxicology	http://www.jsot.jp/english/
Asian Association of Toxicology (ASIATOX)	http://asiatox.com/
Latin American Association of Toxicology (ALATOX)	http://www.alatox.org/
International Society of Regulatory Toxicology and Pharmacology	https://www.isrtp.org/

findings in light of existing regulations. Finally, it is the duty of judges, attorneys, and lawyers to find out the truth.

Like any other expert in a field, a toxicologist must be independent, neutral, and honest. After receiving a formal, often legal request for a statement, toxicologists have to ensure that the issue falls within their scientific and technical competence. If this is not the case, a prompt informative reply to the client is mandatory. Once the toxicologist has accepted a request for a statement, she/he is responsible for the unbiased fulfilment of the job and is solely obliged to provide sound scientific reasoning based on the state of the art. An agreed expert's mandate is generally not negotiable.

Report Types

The past years of toxicological practice have prompted the division of Toxicological Reports into parts: important types are the Toxicological Analysis Report and the Toxicological Assessment Report.

The Analysis Report deals with some specific results, usually of an experimental analysis, but increasingly also of in silico predictions, when computer simulations substitute for expensive, time- and resource-consuming measurements in the laboratory or on-site. When based on experimental data (measurements, observations), the report starts with a short description of the mandate. Next, it includes the (i) circumstances of sampling and specimen conservation; (ii) transport to the analytical site, if appropriate; (iii) handling and preparation steps for analysis; (iv) the analytical method and/or technique applied including negative and positive controls; (v) experimental data of various measurements and observations; and (vi) their statistical significance. Reports are typically prepared by the staff and laboratory experts who performed the analyses and who have a profound knowledge of the applied methodology and potential pitfalls.

The Assessment Report is typically based on various analytical findings or an epidemiological survey on the topic of interest. It starts with a description of the task and elaborates on the circumstances, which prompted mandating of the report. Since toxicological assessment mostly deals with hazards and risks related to human and environmental health, the report usually includes a list of identified hazards and their relevance for the underlying assessment. It then weighs the analytical findings or survey results in the context of available evidence and carefully cites relevant sources and rules.

It is considered good practice if Analysis Report and Assessment Report come from different sites in order to minimize conflicts of interest.

Report Essentials

Traditionally, expert reports consist of at least two parts. Part I is the prologue in which the expert details all the facts that were known to him when arriving at the final statement. These facts include (i) the relevant parts of the disclosed file as received from the court or client, (ii) known facts of the medical history if individuals are involved, and (iii) obtained laboratory results with analytical data and/or specimens, clinical findings, testimonies, and any other relevant evidence.

Part II is the expert's assessment, which is based solely on evidence and findings that were elaborated on in Part I. It is a good habit to begin Part II with the question(s) that were asked to the expert. Any assessment should not come as a plain repeat of evidence and findings presented in Part I. It should also not only reiterate the contents of the filed information. Instead, it should contain the detailed reasoning – step by step – as to how the expert arrived at the

presented conclusions. These conclusions may be given as a statement followed by the relevant arguments and should be based on logical and scientific reasoning.

An optional third part that is highly recommended for complicated or large reports is a short summary in which the essential arguments and conclusions are presented as a concise fact sheet, preferably by repeating each interrogatory issue against the backdrop of the expert's answers.

Toxicological reports should be written in plain language that non-professionals are able to understand. The text should be comprehensible without sacrificing precision. Any special medical or scientific terms should be avoided or should be explained and referenced. This may be accomplished in parentheses or footnotes. For large reports, a glossary may be appropriate, and references should be made to it in the text.

Required Basic Information for Experts

The toxicologist will be asked as an expert about relationships between exposure of one or more individuals to an identified or suspected health hazard or environmental situation. That is why any information about exposure is of utmost importance. It may already be included in the file of the court or client, or it will be determined by the toxicologist, based on his/her own investigations that are clearly presented in the report.

Of similar importance is the determination and assessment of any suspected toxic effects. These may be symptoms, diseases, epidemiologic findings, or environmental abnormalities. If the filed data include observations, statements, expert statements of medical specialists, environmental scientists, etc., these may be included in the toxicologist's reasoning. It is common practice to rank any relationship or association of identified causes and observed effects according to probability. Unfortunately, the English language uses different terms to measure certainty, and toxicologists face a dilemma in putting scientific data into the context of the official language used in court (Brodsky 2009). This is reflected in the three terms "likeliness," "likelihood," and "probability." Table 2 lists the various expressions that are being used to describe any causative or plausible link between an exposure and its outcome.

Table 2 Degrees of likelihood

Impossible	0%
Possible	≤50%
Somewhat probable	60%
Probable	70%
Very probable	80%
With high probability	90%
With utmost probability	99%
With certainty	100%

Reporting Toxicities

One of the most prominent tasks for a toxicologist is reporting toxicities. Whether you think of a new drug or a new consumer product one of the key questions is "How toxic is it?" Considering that a product might consist of several single compounds, the toxicologist faces the challenge to distinguish between the toxicity of each compound alone, any possible combination effects and of course of the entire product itself. Screening for toxic effects has many faces as overt toxicity may occur when the compound is ingested once in sufficient amount, but often repeated doses are required – sometimes in combination with the consumption of other food, e.g., grapefruit – to bring up hidden dangers for human health. In other instances, it turns out that it is not the chemical ingested which causes toxicity but a metabolite that arises from biotransformation in the liver – sometimes only after a critical induction period. Protective regulations have now been implemented in Western countries, which require that one has a rough estimate of the toxicity of the new substance before entering the market. In the USA, two governmental agencies, the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA), take care that new materials are characterized regarding their toxicity before patients and consumers may benefit from their commercial availability. In Europe, the European Union and Switzerland have enforced standards for informing and protecting patients and consumers from various health risks that might ensue from the marketing of new materials. For the EU, the responsible authorities are the European Chemicals Agency (ECHA) and the European Medicine Agency (EMA), while the Federal Office of Public Health (FOPH) oversees new materials in Switzerland. In Canada, the government has Health Canada and the Chemicals Management Plan to protect its citizens, whereas Australia has implemented its National Industrial Chemicals Notification and Assessment Scheme (NICNAS) for similar purposes. Protective regulations are also effective in China, India, and many other countries. So, depending in which country one is to deliver a Toxicology Report, it is wise to contact local experts who are familiar with the effective regulations prior to writing up the Report. In this context, it might be noteworthy that toxic substances "do not know" borders or areas of jurisdiction. Hence, if distinct countries judge the putative harm of a substance differently, the less restrictive jurisdiction might allow the production/release of this substance, which could raise the concentration of this substance in adjoining areas above the limit acceptable to the responsible authorities. Thus, in times of globalization the Toxicology Report should not only address the needs of one jurisdiction but could also consider potential differences with adjoining authorities. This is where global standards come in handy.

ISO 10993 is an important harmonized international standard which regulates the amount and depth of toxicity screening required for approval or certification of medical devices. ISO 10993 lists several endpoints for a comprehensive screening (Table 3) of various devices. Be sure to comply with these regulations before performing and reporting on any testing procedures. In the European Union, all

Table 3 Toxicological endpoints considered in ISO 10993

Cytotoxicity (in vitro)	Sensitization
Acute systemic toxicity (single dose)	Irritation/intracutaneous reactivity
Subacute/subchronic toxicity	Material-mediated pyrogenicity
Chronic toxicity (repeated dose)	Hemocompatibility
Genotoxicity	Implantation
Carcinogenicity	Degradation
Reproductive/developmental toxicity	

knowledge about toxicity of chemical substances is thought to be compiled in the REACH (= Registration, Evaluation, Authorization and Restriction of Chemicals) database which was put into effect in 2007.

ECHA may waive one or more toxicity data from the REACH dossier of a specific substance depending on the annual production and consumption figures, but usually the registrant must include data on various environmental risks (water, soil, air).

A special case of report is the toxicological characterization of natural and synthetic chemical substances in mixtures. This topic is discussed elsewhere in this book.

Reporting Standards for Omics in Regulatory Toxicology

In recent years, it became increasingly important that toxicity, as described in a Toxicology Report, is not a physicochemical attribute of a given substance or mixture but the result of a more or less intense interaction between the substance and a biological organism. Work on 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), one of the most toxic industrial pollutants worldwide, has made this point very clear. TCDD may kill guinea pigs at a single dose of 1–2 μg/kg, whereas almost half of a hamster population may tolerate up to 3000 μg/kg and more (Hengstler et al. 1999). Modern omics (genomics, transcriptomics, proteomics, metabolomics) set out to incorporate that information and put it into perspective. It probably will not be long before qualified Toxicology Report will have to include a section on metabolic profiles to cover these interactions and their implications for human health and consumer safety. Only recently has the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST) initiated a project to develop an Omics Reporting Framework specifically for regulatory toxicology issues. The metabolomics guidelines in the MEtabolomics standaRds Initiative in Toxicology (MERIT) are being advanced in coordination with expert groups to develop the OECD Transcriptomics Reporting Framework and Metabolomics Reporting Framework (Viant et al. 2019).

Cause and Effect Relationships

There are different concepts of cause in various areas of science and jurisprudence. Essentially, jurisdiction and the theory of law differentiate between the theory of equivalence (in criminal law), the theory of adequate causation

(in general civil law), and the theory of essential conditions (social and insurance law). The theory of equivalence describes an action as causal with respect to its success, i.e., the intended action or observed effect, if and only if it cannot be omitted without obviating the specific success. In contrast, general civil law defines an action as causal with respect to its success if the specific circumstances would normally lead to this success, and not some rare, odd, or unlikely peculiarities that are not generally adequate and would have been ignored given the normal (natural) course of things or events. Additionally, the theory of essential conditions requires that a specific cause or event has substantially contributed to the observed "success," e.g., a specific disability.

An example of forensic toxicology may clarify this issue. On a workbench, in his shed, a 60-year-old gardener has kept an aqueous solution of paraquat, a herbicide, in a glass bottle that would normally contain sparkling water. The original labels were removed, and there is a handwritten warning sign, but no other hint of the toxic content. In the absence of the gardener, a 16-year-old neighbor who by chance is the son-in-law of the gardener's sister broke into the secured shed and by mistake took a great mouthful of the toxic water to satisfy his thirst. The ensuing lung edema, followed by a permanent pulmonary damage, eventually caused the death of the boy. The criminal trial is now to investigate the accident in order to determine if a case of familial homicide or a bodily harm with fatal consequences has occurred. The judge may decide that the gardener's action was grossly negligent, because the paraquat solution caused the fatality, and gardener ignored necessary protective measures. Any civil court will approach the case in terms of due compensation, e.g., when asked whether the boy's parents should compensate the gardener for the broken shed door, but may arrive at a similar decision since the break-in is a minor delict that would not normally lead to death, while the toxic drink does. Had the boy survived with a permanent disability, any social court would have attested a reduction in earning capacity. Again, the toxic drink was directly causal and not the illegal break-in.

Occupational and Workplace Issues

Within a forensic framework, the court will define the precise questions that the Toxicology Report must deal with. Therefore, it is mandatory that the expert strictly adheres to these specific questions. A rather complicated issue is the elaboration of a causal medical assessment in terms of the social law. Quite often, this question arises if the underlying cause is an occupational illness. In such cases, insurance companies, e.g., the Accident Prevention and Insurance Association, may be obliged to provide fair compensation for health care and rehabilitation. Recognized occupational diseases are overseen by the International Labour Organization (ILO 2010). An occupational disease is accepted if the medical diagnosis has been confirmed, the exposure conditions have been unequivocally verified, and the medical evidence confirms cause and effect. The latter is usually agreed on if the causal relationship between exposure and disease has been assessed as probable. That is, there is more reason to argue in favor than against the assumed relation of cause and effect. Any

Toxicology Report that deals with occupational and/or workplace issues must reflect these considerations and provide a provisional answer about the likelihood that identified hazards and observed damages of health and safety are linked.

With regard to liability, law systems are quite different among countries of the European Union and may even be more different between the so-called G8 nations and other countries of the world. Employees may be protected against health injuries from workplace exposure, but to varying degrees. In the USA, the Division of Federal Employees' Compensation (DEFC) at the US Department of Labor will oversee compliance with the Federal Employees' Compensation Act (FECA) through its district offices, which are located throughout the country. In principle, the act treats permanent and temporary employees alike. However, the employee must provide medical and factual evidence to establish the essential elements of the claim, i.e., that the claim was filed within the statutory time requirements of the FECA, the injured or deceased person was an employee within the scope of the FECA, the employee suffered from an injury or disease, the employee was in the performance of duty when the injury occurred, and the condition in question resulted from the injury. If the injury has not been reported, no benefits will be paid. The restrictions on these reports are rather strict: If medical treatment is required, a special form has to be submitted – usually within 4 h of the request. Retroactive issuance is not permitted under any circumstances. If the damaging event was a toxic substance, the toxicologist will have to fill in the gaps and answer the questions put forward by the district officers. In Germany, for example, social courts have to decide if an employee will receive compensation for an acquired disease that has been linked to workplace exposure. If the disease is on the list of known occupational health problems, the Accident Prevention and Insurance Association ("Berufsgenossenschaft" in Germany) will cover any illness-related expenses (for therapy and rehabilitation, if appropriate). If the disease is not on the list, the worker's health insurance would have to pay the costs to restore the worker's health. Once a causal relationship between a toxic exposure and an observed disease has been established, but the disease is not (yet) covered by the International Labour Organization, there is an exemption clause that will allow the worker's condition being treated as an occupational disease although it has been shown that notification of an occupational disease may increase the risk of work disability (Kolstad et al. 2013). In order to do so, four criteria have to be met: (i) a special group of workers or employees has to be identified that will be exposed to certain influences at the workplace more seriously than the general population and the affected person, i.e., the patient, must be a member of that group; (ii) the claimed influences must be able – according to the state of the art and the medical science – to cause the observed health injury; (iii) scientific knowledge about the newly suspected influences was not available or insufficient, when the last revision of the list of known occupational disease was made, or has not been thoroughly checked; and (iv) a causal relationship between worker's or employee's duties and the ensuing illness must be a "deemed-to-satisfy" provision. Once again, the toxicological expert will have to provide evidence or deny such a link, before the patient will be promoted to an applicant for compensation. The expert has to deliver his/her scientific opinion for each of the four points, and the

statements must respect the available literature in the fields of occupational and social medicine. If the expert fails at any point, chances are that the whole report with all its arguments will be dismissed in court. Conversely, a carefully written report with convincing data and evidence may provide enough reason to resume hearings and discussions that may eventually lead to the filing of the newly discovered condition as an occupational disease.

Adverse Drug Effects and Drug-Related Disease

For a long time, adverse drug effects have been recognized as inevitable sequelae of certain medical treatments. Modern drug safety requirements and consumer protection regulations require that such information be provided to the patient on a case-by-case basis. But drug makers continue to collect data even after their products enter the market and such data-on-file dossiers may provide an important source for keeping the quality of information up-to-date. Consequently, Toxicology Reports in special formats, e.g., Warning Letters, Rote-Hand-Briefe, and others, continue to be an essential part of drug surveillance, which reflects the progress being made with an increasing number of observations (Fig. 2). It should be noted here that data integrity, i.e., the inclusion and labelling of info data according to the ALCOA principle (= attributable, legible, contemporaneously recorded, original (or a true copy), accurate), has become increasingly important for audits and reports, likewise (Food and Drug Administration 2018).

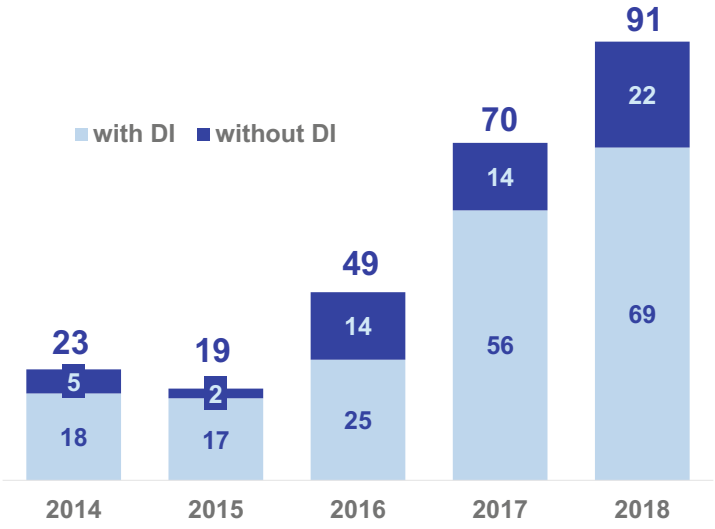

Fig. 2 Global drug manufacturing letters by data integrity. The issue of data integrity (**DI**) has become increasingly important over the years as shown by this North America survey. (Modified from: de la Torre 2019, available from: https://govzilla.com/blog/2019/05/pharma-medical-devices-data-integrity-breaking-down-keywords-and-citation-trends-from-the-fda/)

The physician who prescribes a certain drug is usually held responsible for informing the patient. It should be emphasized that despite potentially severe adverse effects, drug treatment is warranted if the benefits for an individual outweigh the risks. Recent trends appear to enforce the patients' rights of clear and understandable information about the most frequent and (or) most serious adverse drug effects. Failure to provide this clarification at the beginning of treatment may be sued as medical malpractice. Nowadays, toxicologists may be asked for their scientific opinion in court. In such cases, it is mandatory that the expert is unbiased and independent, scientifically sound and has no personal history that might be deemed a conflict of interest.

Work Disability and Reduction in Earning Capacity

One of the issues most often dealt with in Labor Courts is the reduction of earning capacity. Even minor shifts in the granted percentage will have great repercussions on the patient's compensation and benefits. However, the toxicological expert should be aware that clinicians and specialized physicians generally provide such an assessment. Only in exceptional cases will the toxicological expert be asked for some scientific statement. The most notable exception is occupational cancer and carcinogenesis when the toxicologist should provide his/her expertise and give an estimate of likely sequelae and prognosis, based on the most recent research and study results in the field of interest.

Historical Crosslinks to Regulatory Affairs

The above given examples should have made clear that Toxicology Reports may have a strong impact on later amendments of jurisdiction. For example, a new entity may be added to the list of occupational diseases if an increasing number of expert reports emphasize its relevance and the experts communicate the need for such a revision. In Germany, the Contergan Trial (1968–1970) was the most prominent example of how toxicological experts might take part in the decision-making processes in politics and public opinion and thus trigger a fundamental revision of the country's law (Stephens and Brynner 2001). The German Medicines Law, which became effective in 1971, was significantly influenced by the science of toxicology, by toxicologists and other experts in the field, and reciprocally stimulated toxicological research in Germany and elsewhere.

Recommended Internet Sources

As stated above, there is no generally agreed standard on how to report the various aspects of toxicological screening for scientific and forensic purposes. But there are several resources in the worldwide web from various organizations, which strive for the improvement of report quality, and standards provide suggestions and templates

to harmonize reports. The entries that are listed in the paragraph "Resources" are by no means complete but provide a starting point for everyone who is in the need of making contributions in scientific meetings, to regulatory authorities, and in court. Many sources do not only outline how to report but rather focus on the conduction of proper toxicity screening procedures for which the report is a final step when presenting the results to the public.

Resources

(i) Templates for reporting various aspects of toxicology screening of chemical substances have been formulated already in 2003 and can be found at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-templates-reporting-toxicology-data.
(ii) Recommendations for reporting forensic content have been published as drafted ASB Standard 053, First Edition 2018 by the AAFS Standards Board (available at https://asb.aafs.org/wp-content/uploads/2018/09/053_Std_Ballot01.pdf)
(iii) A useful collection of documents category descriptions can be found at the National Institute of Standards and Toxicology (https://www.nist.gov/topics/organization-scientific-area-committees-forensic-science/standards-and-documents-category)
(iv) ECHA: Practical Guide 14: How to prepare toxicological summaries in IUCLID and how to derive DNELs http://echa.europa.eu/documents/10162/13655/pg_14_on_hazard_endpoint_en.pdf.
(v) Colorado Business and Legal Advices (©2011 Colorado Business and Legal Advices) http://www.partidocolorado.org/individualizing-theories-theory-of-the-adequacy-and-adequate-theory-of-causation.htm.

Further Reading

For an in-depth information on the ISO 10993 regulations see http://nhiso.com/wp-content/uploads/2018/05/ISO-10993-11-2017.pdf

For more information on the role of the toxicology expert the reader is referred to Wall's book on expert witnesses (Wall 2009). Experts had a long time as "immune" participants in court trials, but this is no longer true for many countries including the US Forrest 2014.

Cross-References

► Importance of Xenobiotic Metabolism: Mechanistic Considerations Relevant for Regulation
► National and International Collaboration in Regulatory Toxicology

► Omics in Toxicology
► Single Substances Versus Combined Effects: Problems in Mixture Toxicology
► Toxicological Risk Assessment in Different Jurisdictions

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Risk Management in Toxicological Disasters 72

Kai Kehe, Dirk Steinritz, and John H. Duffus

Contents

K. Kehe (✉)
Department F – CBRN Medical Defense, Military Medical Research and Development, Bundeswehr Medical Academy, Munich, Germany
e-mail: kai.kehe@lrz.uni-muenchen.de

D. Steinritz
Department F – CBRN Medical Defense, Bundeswehr Medical Academy, Munich, Germany
e-mail: dirk.steinritz@lrz.uni-muenchen.de

J. H. Duffus
Toxicology, The Edinburgh Centre of Toxicology, Edinburgh, Scotland, UK

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Abstract

Toxicological disasters, such as industrial accidents or the use of highly toxic compounds in terroristic attacks or civil wars, are rare, but more frequently occurring events with high impact to health and environment. Medical preparedness in such situations requires policy, planning, and a preorganized response system on a cross-ministry basis. This chapter briefly describes the main parts of the planning process and the needed legal framework to support this process. Production, storage, transport, use, etc., should be monitored to prevent or minimize the chance of release of hazardous substances. Knowledge about toxicological profiles, characteristic symptoms of poisoning (so-called "toxidromes"), and medical countermeasures (including stockpiling and availability of antidotes) should be readily accessible. The response system has to provide detailed plans for the management of toxicological events and exercise the procedures on a regular basis involving all levels of command. Financial support is mandatory to support continuous toxicological research, to build up and maintain the working capacity of poison control centers and specialized medical response teams. These efforts taken together should form an efficient risk management system.

Keywords

Toxicological disasters · Terroristic attacks · Framework · Preparedness

Introduction

The release of poisonous substances, either accidentally or intended, can cause severe health effects in a huge number of people and can result in disaster. For example, ergotalkaloids caused mass poisoning during the middle ages. More recently, the extensive use of chemical warfare agents during World War I and the Iraq–Iran war (1980–1988) caused hundreds of thousands of soldiers to be injured. Terrorists from the Aum Shinrikyo Cult released sarin in Matsumoto and Tokyo in 1994 and 1995. The Tokyo subway attack resulted in 12 fatalities and 980 injured persons. More than 5,000 people thought they might have been poisoned (so-called "worried wells") and consequently sought medical help. In 1984, 42 tons of methyl isocyanate leaked into the air from reservoirs of a local chemical plant in Bhopal. There were 5,000 fatalities and more than 200,000 long-term injuries counted within an area of 20 km^2. The confirmed use of chemical weapons in the Syrian Civil War is the most recent incident that documents the deliberate use of chemical warfare agents against the civilian population. These examples clearly demonstrate the urgent need for preparedness, including planning and organization, as well as availability of appropriate medical countermeasures.

The onset of acute clinical symptoms after the release of toxic substances may vary between seconds and days depending on the nature of the poison. In addition to

acute health effects, symptoms with a delayed onset may occur. Moreover, even a single exposure event may provoke long-term effects.

From a medical perspective, a disaster situation has to be expected in mass casualty events that either overwhelm the local community's medical services and/or result in contamination of relevant medical infrastructure. This means that the relationship between the capacity of local response teams and their need for external aid is more important to qualify an incident as a disaster than is the total number of intoxicated persons or fatalities.

Characteristics of Toxicological Disasters

The release of a toxic chemical may only be realized when a high number of patients present with comparable clinical symptoms. Initially, the exact toxic compound may be unknown. In this phase, risk assessment and patient treatment are based on clinical signs and symptoms. However, unambiguous identification as fast as possible by using appropriate detectors or test systems may help to readjust risk estimation and medical countermeasures. Especially the release of complex mixtures of toxic gases (e.g., during fire accidents) necessitates rapidly available analytical methods close at hand. A contamination of the ambient atmosphere, soil, buildings, and persons has to be considered unless proven otherwise. For this reason, personal protective equipment is essential to enable rescue operations within the contaminated area. However, wearing personal protective equipment (PPE) significantly limits the action time and requires replacement of personnel dependent on the environmental condition. In the worst case, the action time may be limited to less than 1 hour. Thus, medical treatment of injured and (or) intoxicated patients is severely impaired.

Secondary contamination of medical personnel and infrastructure may be accelerated by the evacuation of contaminated patients, animals, or traffic through the hot zone. Thus, all potentially contaminated persons and materials have to be thoroughly decontaminated before leaving the hot zone. In order to avoid uncontrolled trafficking, it may be required to control and secure the hot zone by security forces.

A decontamination line in front of hospitals or other relevant infrastructure is mandatory to protect these.

Preparedness, Regulatory, and Legal Frameworks

Massive release of toxic substances affects both environment and infrastructure. Thus, a wide range of capabilities is necessary to cope with a toxicological disaster. Local authorities and communities are responsible to provide the required plans of operation. There are legal requirements to ensure proper planning and training of local public health service, agencies for technical relief, fire and ambulance services, police force, and homeland defense to respond to and to manage the incident scene in its early phases. In later phases, more specialized help (both civilian and military) is needed to provide more sophisticated expertise. The legal framework to manage this kind of disaster differs

from nation to nation and even within a nation. Because of this, delays may occur that may complicate the situation. Continuous political leadership is the only chance to prevent these issues. In times where homeland defense is reduced in several countries due to tight budgets, it is important to enhance risk perception so that the high-risk areas are given priority for appropriate control measures to be taken (OECD 1994).

A consistent legal framework of responsibilities, tasks, procedures, alarm plans, and communication lines is crucial for the successful management of disasters or catastrophes. The availability of points of contact and key personnel 24 hours and 7 days a week is essential.

Training, Drill, and Exercises

Efficient collaboration between different agencies, governmental (GO) and non-governmental organizations (NGO) depends on their current knowledge and practical skills. Regular exercises including the highest political levels are necessary to achieve an appropriate level of preparedness and understanding. All participants must be aware and prepared for their tasks. Such preparation necessitates a profound understanding of toxicological risks and available countermeasures. Appropriate protective equipment must be available in sufficient quantity to deal with any possibility of a major disaster. Personnel must be trained on a regular basis to ensure safe handling of protective equipment.

Identification of Potential Hazard Sources

To counteract potential terrorism, integrated intelligence systems with interagency activities are essential to recognize threats as early as possible. This includes not only knowledge about the capabilities of a potential antagonist, but also identifying industrial facilities that store and produce substances that might be of interest for terrorists. Additionally, transport routes should be known and analyzed for potential risks. Regulatory approaches that may be applied include the UN Recommendations on the Transport of Dangerous Goods, Dangerous Goods Emergency Action Code List 2011, and Canadian Transportation of Dangerous Goods Regulation. To supplement the above activities, an epidemiological surveillance system should be introduced for early detection of the effects of any hazardous substances that may be released into the community.

Identification of Hazards Through Emergency Forces

Toxic chemicals (solids, liquids, or gases) that can harm people, other living organisms, property, or the environment are classified as dangerous goods. Regulation of such chemicals should be enforced by local regulatory agencies. Proper labeling of chemicals is essential for the safety of emergency forces and to

ensure proper countermeasures. The Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) is a European Union regulation to enforce the safe use of chemicals. A comparable law is the US Toxic Substances Control Act. As described in the previous section, the transport of dangerous goods is strictly regulated at both international and national level. Especially first responders must be familiar with the legal framework and the labeling system. In addition, an analytical task force should be available to identify unknown chemicals and to ensure that the correct measures are taken to minimize their potential to cause harm.

Detection

Various devices are available for the rapid detection of unknown chemicals. However, detection systems have been optimized for a number of civil or military scenarios, and unfortunately, there are always new and unforeseen events occurring. Thus, it is essential to have available first-class and up-to-date analytical chemists and a well-equipped laboratory in order to devise and apply new methods which may be necessitated as new problems are presented. To ensure reliable results, sample collection and preservation (e.g., with refrigeration and lightproof containers) must be properly carried out. Transportation to the analytical laboratory must be as quick as possible. In addition to the addresses of the laboratories that are involved in normal emergency response, emergency response units should also keep a list of addresses of laboratories with special knowledge and skills (e.g., universities, industry, and the armed forces).

Information on Toxic Substances

Companies that use highly toxic substances should develop a risk management program (RMP). Information, including material safety data sheets, should be available for protective, diagnostic, and medical countermeasures. Poison control centers should have this information available because they have a central role in the initial planning of medical countermeasures. Specialized knowledge about chemical warfare agents will be available from military sources. Specialized internet databases may also provide detailed information. Regulatory laws under the European REACH regulation should eventually ensure the availability of all relevant data regarding industrial chemicals.

Limits of Exposure

A number of exposure limits have been defined for the safety of human beings, workplace, buildings, and environment. These limits are of minor importance during the emergency management of toxic catastrophes. However, they are helpful to estimate limits for acute short-time exposure for emergency personnel during the initial phase of disaster management. Additionally, they are useful for information of exposed

population about possible hazards (OECD 1994). These limits become highly relevant in the aftermath of the accident in ensuring that harmful consequences are kept to a minimum.

Physical Protection

Toxic contamination of air and body surfaces requires physical protection of airways, eyes, and skin. Without certain knowledge of the nature of the released toxic substance, it is necessary to wear whole body protection and a self-contained breathable air supply. Airway protection may be sufficient in some cases if the potentially toxic substance involved has been identified. Masks, suits, gloves, and overshoes should be made of appropriate material with a high protection factor. In order to choose the right equipment, it is necessary to have data readily available defining relevant protective properties (Gupta 2020).

Decontamination

Contaminated persons have to be decontaminated before leaving the hot zone. It is crucial that intoxicated persons do not contaminate hospitals or other critical infrastructure. The preparedness of hospitals and the public medical service is a legal requirement in some nations. Sufficient amounts of clean water and decontamination equipment should be available. Management of wastewater must be considered as well. Hospital emergency plans should contain information about the traffic routes to and from the medical facility, and hospitals should have sufficient stockpiles of antidotes, beds, and blankets (WHO 2004).

Drugs

Life-threatening poisoning is a rare event. Usually, stockpiles of specific drugs and antidotes to treat intoxicated patients are small and not sufficient to cope with a mass casualty situation. Lifesaving antidotes should always be kept available for emergency personnel to treat contaminated patients but also for self-treatment after self exposure, e.g., by damage to their protective equipment. Further stockpiles of antidotes and other drugs must be available at short notice. Distribution depots should be established, and their location must be known to ensure short transportation times. Pharmaceutical companies should participate in planning for the rapid supply of necessary drugs.

Centers of Expertise

Centers of expertise for the management of toxicological catastrophes are rare. They may be part of a ministry, university, company, or even of the military. Laws should ensure that a sufficient quantity and quality of toxicological expertise are available on a national level. This expertise is especially needed after a toxicological incident and should be responsible for human biomonitoring to follow up exposed patients.

Research and Development

Risk assessment, preparedness, and strategic planning are the responsibilities of national authorities. However, basic scientific expertise is needed as well as capability to respond to an incident. In order to sustain toxicological research, adequate financial support has to be secured as well as the establishment of research institutes at the medical faculties of leading universities. Medical students and doctors should be supported to participate in toxicological research programs. These programs are mandatory to support the education of new scientists with a medical background. This future scientific expertise is necessary to support ongoing research. Currently, a small number of scientists have to deal with a plethora of urgent toxicological problems:

- Development of new antidotes
- Development of a system to decontaminate injured patients
- Development of a decontamination treatment which can be applied to eyes and mucous membranes
- Development of new methods to analyze and monitor toxicological exposures

Table 1 Management in toxicological disaster – some essentials

Preparedness	Incident management	
Legal framework	*Hazard identification*	*Protection measures*
Training, drill, and exercise		
Key stakeholder organizations	Support by emergency forces	Physical protection
Analytical equipment	Support by poison centers	Decontamination
Stockpiles	Source identification	Antidotes & medical countermeasures
Access to toxicity data & guidelines	Sampling	Evacuation procedures
Documentation SOPs	(Forensic) Analysis	Documentation SOPs

Risk Management

WHO suggests the implementation of a structured planning process to meet the needs of effective risk management. The following step-by-step approach is suggested:

1. Identify the hazards
2. Evaluate the hazards to determine the probability and severity of the initial risk
3. Introduce risk-reduction strategies
4. Quantify the residual risk, and decide what risk is acceptable
5. Monitor the risk management program and repeat the process as required

Summary

Some major elements of toxicological disaster management are shown in Table 1.

Cross-References

- ▶ Chemical and Biological Weapons and Their Regulation
- ▶ Dealing with Diseases That Have Been Attributed to Chemical Exposures
- ▶ Health Hazard Classification and Labelling
- ▶ Prohibition and Restrictions in Regulatory Toxicology
- ▶ REACH and CLP: Its Role in Regulatory Toxicology

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Institutionalized Participation in Regulatory Toxicology

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Andreas D. Kappos

Contents

Abstract

Public participation in risk management decisions has a sound legal base in Europe. In democracy it replaces the authoritarian top-down risk management. Participation of the public changes the role of scientific toxicology. Toxicologists should state clearly their models, assumptions, and resulting uncertainties and strictly separate scientific analysis from extrapolation and opinion. Participatory risk management is based on the notion of the emancipated citizens, who are capable of informed risk appraisal and enable them to contribute their interests,

A. D. Kappos (✉)
Formerly: Department of Health and Environment, Hamburg Health Authority, Frankfurt, Germany

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beliefs, and values. The rationale and supposed benefits of public participation in decisions about environmental health risks are discussed, and different models applied so far are presented.

Keywords

Public participation · Risk management

Introduction

The *Aarhus Convention* of 1998 (UNECE 1998), signed by 40 countries and the European Union, guarantees the rights of the public to participate in decision-making in environmental matters. Substantial evidence shows clearly that an adequate involvement of *stakeholders* and persons concerned in environmental health decisions achieves more effectively better and enduring results. Goals of participation should be to de-emotionalize conflicts, to reconcile different points of view, and to develop a common basis of assessment and consensual strategies for solutions, which do justice to the distinct interests of the different stakeholders. Participation enables better acceptance of decisions. The unsolvable points of conflict may be unearthed, and last not least the transparency of political decisions increased.

Why Participation?

In the classic concept of "*top-down*" *risk management*, public administration regulates risks for the public or for single individuals using adequate measures, which in their discretion best suits the problem. The background of the decision, such as the scientific facts and their *uncertainties*, the *conflicts of interest*, the accepted compromises and weighing processes, and the remaining residual risk, remains hidden to the public. The role of the toxicologists as a scientific expert is to propose suiting treatment policies to the administration. Their expert statements serve to justify decisions that are founded on scientific facts, but depend as well on *normative values*. The underlying assumption of this risk management model is as follows: The administration well intentioned represents public welfare whereas stakeholders want only to enforce their special interests.

In the 1970s and 1980s, sensational incidents and in hindsight obviously wrong risk assessments by governmental policy makers scandalized by the media put public confidence in the ability of the administration to regulate risks for the benefit of the general public seriously in question. People's trust in the impartiality and objectivity of scientific risk statements was lost. Affected parties demanded loudly a participation in risk decisions. The political implementation of controversial techniques, such as genetic engineering or waste incineration, got almost impossible if they were felt threatening, justified or not. Small risks were overregulated, large neglected. A rethink was strongly indicated.

Participatory Risk Management

Social science risk research indicates that the *rating of environmental risks* on human health depends not only on its height but at least equally on the uncertainty of the scientific facts and on the values the rating persons have. In a society, in which pluralism of values prevails, risk assessment and management must be democratically legitimized. The paradigm of risk management as a participatory process in mutual faith replaces the "top-down" model of risk management. It is characterized by a broad participation of all people concerned, the so-called stakeholders.

In this counter-model of a *participatory risk management*, the authorities provide basically the framework for process-oriented regulations. Decision processes take place as locally as possible, if possible at the local level where the risks occur. Science is not presented anymore as the single factor determining the decisions about risks, but as one factor among others, such as economic criteria and value-led trade-offs. Science is accessible to all people involved.

Participatory risk management changes the role of scientific toxicology. Toxicology is required to adhere strictly to scientific data and to refer only to facts that are proven according to sound scientific methodology. On the other side, scientists have to be explicit about the extent and limits of their knowledge. They should state clearly their models, assumptions, and resulting uncertainties and strictly separate scientific analysis from extrapolation and opinion. For the public, it is critical to know not only what definite knowledge is but also what is still ambiguous.

Participatory risk management is based on the notion of the emancipated citizen, who is capable of informed risk appraisal. The concept means being in a position to make an informed risk appraisal on the basis of knowing the objectively demonstrable consequences of risk-generating events or activities, the residual uncertainties, and other risk-relevant factors and to rate the risks according to the individual's values for shaping his own life and in correspondence to his personal criteria for assessing the acceptability of these risks for society as a whole. Once this capacity for *informed risk appraisal* on the part of the citizen is acknowledged, it is the task of the authorities to build up and maintain the communication base necessary for this purpose. In the context of risk communication, there is a need for all forms of communication, from simple documentation of results, through targeted information offerings, to forms of dialog and of participation in the decision-making process.

Legal Background of Participation

In democracies the participation of each person in decisions that affect them is viewed as a fundamental right. In 1989 all the Ministers of the Environment and of Health of the European region of WHO signed the *European Charter on Environment and Health* (WHO-Euro 1989), which states: Every individual is entitled to . . .information and consultation on the state of the environment, and on plans, decisions and activities likely to affect both the environment and health (and to) participation in the decision-making process.

Principle No. 10 of the Rio Declaration (UNEP 1992) declares that environmental issues are best handled with participation of all concerned citizens, at the relevant level. At the national level, each individual shall have appropriate access to information concerning the environment that is held by public authorities, including information on hazardous materials and activities in their communities and the opportunity to participate in decision-making processes.

These fundamental rights were materialized in the UN/ECE Convention on Access to Information, Public Participation in Decision-Making and Access to Justice in Environmental Matters (UNECE 1998), signed 1989 in Aarhus, Denmark, by more than 40 states and the European Union, the so-called *Aarhus Convention*. For the EU, the institutions (European Commission, European Parliament, European Council, etc.) are bound by the convention through the so-called Aarhus Regulation. In the framework of EU legislation, rights of the public to be informed and to participate in decisions are guaranteed in the *EU Strategic Environmental Assessment Directive* 2001/42/EC (EU 2001) and the *EU Public Participation Directive* 2003/35/EC (EU 2003). Both directives are binding on the Member States and accordingly have been implemented into national law.

However, in 2017, the Compliance Committee of the Aarhus Convention ruled that the Aarhus regulation of the EU does not give people and NGOs enough rights, and in many EU-countries there are too many constraints that limit proper participation (Pánovics 2017). Lately a tendency is observed to restrict public participation in order to speed up planning processes.

On the other side the European Food Safety Authority established in 2003 a specific Stakeholder Forum to include contributions of consumers and industry into their decisions and the European Chemical Agency launches calls for comments and evidence in the preparatory phase of the restriction proposal and on the different documents under preparation in ECHA in relation to restrictions, such as reports on substances in articles and guidelines on restriction entries. Anyone can submit comments.

Rationale and Benefits of Public Participation in Risk Decisions

The benefits of *stakeholder participation* in risk decisions are obvious (Table 1). The participation of people concerned by risks or generally interested in risk issues should eliminate the widespread suspicion of the public towards authorities and established science. It should ensure the transparency of the foundation, framework, and underlying assumptions of the decision and finally promote their acceptance. For the authorities, participation of the public provides opportunity to become acquainted with the fears and worries of the citizens and their specific concerns, which they can take into account. Local experience and knowledge can be utilized for risk management. Finally, public participation encourages the participants to focus on arguments rather than on ideological contradictions. Timely involvement of the persons concerned may possibly avoid time-

Table 1 Benefits of stakeholder participation in risk management

Democratization of the decision processes
Inclusion of different values in society
Promotion of a better understanding of administrative decisions by the public
Improvement of the knowledge base
Saving of time and costs
Trust building
Supporting acceptance of decisions by the public

consuming legal disputes and heavy conflicts fought out in the media or in the political arena. However, participation is no panacea. Objective clash of interests cannot always be settled, and unsolvable conflicts must ultimately be decided at the political level.

Participation adjusts to the three key challenges of rational risk management. What issue dominates a specific process and defines what type of participation is most useful. If the complexity of scientific data as to the cause-effect relationship prevails, then the main issue will be clarification and explanation of difficult-to-understand scientific facts to lay people. Main conflicts arise at interprofessional level. The objective of public participation in this case is to inform about the scientific facts and make the scientific debates transparent to the public. Thus, it serves the understanding between experts and laymen.

If predominantly the *uncertainty* about the level of risk is under debate on account of methodological uncertainties, statistical variability or limitations, and uncertainty of scientific knowledge, then it will be important to find the narrow path between excessive caution and irresponsible negligence. Risk benefit considerations may be the remedy of choice (see ▶ Chap. 59, "Risk-Benefit Considerations in Toxicology"). A balance must be struck between the burdens of those who have to bear the risk and the benefit of those who create the risk.

If *ambiguity of risk*, which means different interpretation and evaluation of scientific facts according to differences in values, is the issue, then the acceptability of risks has to be negotiated and finally decided. Cultural, social, and ethical values have to be taken into account. In this case participation serves to improve understanding of different positions and to guarantee a fair and equitable procedure.

Who Should Participate?

The process should include credible representatives of the full spectrum of parties, who are interested in or will be affected by a decision (Table 2). It should be structured to encourage their voluntary commitment. Basically, anyone who feels affected by a specific risk has the right to participate. In practice only few people will have such an immediate interest to sacrifice time and money required to fully participate in the decision process. Such people feel personally affected in their

Table 2 Stakeholders in participatory risk management

Local initiatives concerned with the risk issue
Representatives of cultural, ethnic, or economic groups and associations
Local authorities
Public health service
Industry and chambers of commerce, business associations
Local practitioners and their association
Trade unions
Environmental associations
Relevant research institutions
Institutions responsible for standard setting

lifestyles, their health, their economic interests, or their values and organize themselves in grassroots initiatives. But this must not lead to the fallacy that grassroot initiatives represent only the interests of a small minority. Experience has shown that risk managers often be wrecked, if they doubt whether these initiatives represent the general public and if they try to play the so-called silent majority against them.

In order to decide, who has to be involved, authorities have to ask the questions: Who is affected by the risks (but also of the measures to eliminate the risks)? Who has additional information or expertise? Who was affected by similar risks in the past? Who could be upset if not invited?

Models of Participation

In the past various *models of a public participation* have been applied.

Publication of Decision with a Set Period to Submit Objections

The decisions of the authority are made accessible to the public. Everyone can raise written objections within a prescribed period. The authority must deal with them. Participation is aimed mainly at professionals and associations.

Hearing

Similar to the previous model, arguments from interested parties could be raised and publicly discussed with the authority. In this and the previous model, the objective is to bring arguments to the authority's notice, which was not considered in the initial decision. According to all experience, the effect on the final decision is low, because the authorities are not bound to take additional arguments into account. Usually the participation takes place at a time, at which the cause has largely decided and authorities hate to revise once taken decisions. Hearings are legally prescribed in a

Table 3 Five key principles for effectively melding scientific analysis and public participation

1. Ensuring transparency of decision-relevant information and analysis
2. Paying explicit attention to both facts and values
3. Promoting explicitness about assumptions and uncertainties
4. Including independent review of official analyzes and/or engage in a process of collaborative inquiry with interested and affected parties
5. Allowing for iteration to reconsider past conclusions on the basis of new information

number of European environmental laws. Participation is typically restricted to persons or institutions with a "legitimate" interest on the issue.

Round Table

The experience that hearings in practice contribute little to a de-escalation of risk-related conflicts leads to the establishment of so-called round tables in particular settings. The objective of this exercise is to negotiate with as many opponents, as possible, at an early stage of the decision process with the hope that the final decision will be accepted by most of the stakeholders. Critical for the success of a round table is the inclusion of all people concerned, a collaborative formulation of the problem, a good faith communication, and last not least the transparency of all decision-relevant information. Specifically the role of scientific expertise has to be considered carefully and accessible to all participants. As put by the Panel on Public Participation in Environmental Assessment and Decision Making of the US National Research Council generally (NRC 2008), it should be wise for responsible agencies to follow the five key principles shown in Table 3.

From (NRC 2008)

Advisory Board

An Advisory Board is made up of representatives of the interested parties and experts and accompanies a planned project from the beginning. The Board must have clearly predetermined competences to interfere with the decision process. In practice the delegated members of the advisory board are endangered to decouple themselves increasingly over time from the interest of their base.

Mediation

In addition to the participating interest groups of the "round table," an impartial arbitrator (mediator) is appointed to guarantee a fair deliberation. Its role is to promote the integration of diverging positions. So to speak, the mediator should act as a catalyst for consensus. Mediation is indicated, where conflicts between stakeholders are evident in the run-up of risky projects.

Table 4 Models of public participation in risk management: pros and cons

Participatory models	Pros	Cons
Publication with objections	Transparency	Minor influence on decision
Hearing	Transparency, platform for diverging arguments	Minor influence on decision, no settlement of conflicts
Round table	Fairness	Time-consuming questionable legitimacy
Advisory board	Anticipated settlement of conflicts, expertise, competence to decide	Limited participation, questionable legitimacy alienation from public
Mediation	High potential to settle conflicts, fairness	Time consuming, low efficiency
Cooperative discourse	Adequate to the problem, effective, efficient	Costly

Cooperative Discourse

This model, proposed by (Renn 1999), consists of three steps: identification and selection of concerns and values of the stakeholders, identification of impacts and consequences of different policy options by experts, and finally evaluation of potential solutions by a panel of randomly selected citizens. In the last step, the stakeholders and the experts contribute only as witnesses. They provide their arguments and scientific evidence to the panel, which ultimately decides on the various options. In this model the phases of elicitation of scientific facts, elaboration of deviant values of people concerned, and weighing of facts and values are clearly separated into three panels with varying participants.

Pros and cons of the different participatory models are summarized in Table 4.

Evaluation of Participation

In spite of the legal establishment of public participation, its application in practice has been criticized repeatedly (SRU 2002; NRC 2008; Pohjola and Tuomisto 2011). But the evaluation of public participation has been focused on process and access rather than on outcomes. But what is important from the point of view of participation in risk assessments and management is the influence allowed for the stakeholders in the different settings. The framing of a risk assessment approach can be a significant constraining factor for potential effectiveness of participation. For example, the commonly applied approach to environmental health assessment treats stakeholder involvement and public participation rather as an add-on brought about by legal requirements than as an essential aspect of risk assessment or decision-making. The common current practices of participation are not necessarily always in line with the latest

discourses in the literature, and the law seldom requires very high degrees of openness to public participation. It is usually built on the conventional frameworks of administrative decision-making. Professional risk assessors and policy makers fear losing their power, although they should see themselves as feeding an open collaborative process with their expertise. Pohjola et al. addressing the issue of effective participation developed a concept of five dimensions of openness of risk management, which include the scope of participation, access to information, timing of involvement of the public, aspects of the issue the participants are allowed to contribute, and the weight given to the contributions in the final decision. The framework of openness provides a context for evaluation and constructive criticism of contemporary institutions and practice of public participation in risk assessment, and policy making, and a basis for developing new models. Openness should not, however, be considered as an end in itself, but rather a means for advancing societal development through creation and use of broadly distributed collective knowledge upon issues of great societal relevance (Pohjola and Tuomisto 2011).

Conclusions

Participation in risk assessment and policy making upon issues of environment and environmental health has become a commonplace. There are numerous good examples of public participation to manage risks at the local level. At the national or international level, specifically for the management of toxic substances in consumer-relevant products, they are scanty or completely missing. Existing experience with participatory risk management shows that participation can be an invaluable part of risk assessment and decision-making. There are no simple "best practices" that provide universal guidance (NRC 2008). Therefore, the creativity of risk managing authorities is challenged.

The strength of participation is that aided by discursive methodology, it is possible to weigh arguments by rational and political legitimized criteria before deciding. A formally structured and organized deliberate procedure takes notice of scientific expertise, laws, norms, social interest, and people's values. If it is conducted in a fair and representative manner, it will integrate rational, emotional, and normative statements and opens a perspective to solve conflicts of interest. Prerequisite is the willingness to debate, to learn, and to compromise.

It is not possible to predict in advance how effective a particular participation process will be to avoid or lessen conflicts. Participation has limitations. It is reasonable only if there is still something to decide and if willingness to compromise exists on the side of all parties involved and last not least if the public is supported by trustworthy scientific experts. Participation is not effective, if it depraves to unlimited debating. Besides well-intended openness, the majority of concerned citizens will always remain bystanders.

Cross-References

- ▶ Risk Communication: Challenges for Toxicologists and Other Risk Experts
- ▶ Risk-Benefit Considerations in Toxicology
- ▶ The Regulatory Process in Toxicology
- ▶ Uncertainty Analysis in Exposure Assessment-Relevance for Toxicological Risk Assessment

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Risk Communication: Challenges for Toxicologists and Other Risk Experts

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Werner Lilienblum and Marianne Lilienblum

Contents

Abstract

This chapter is primarily written for scientists concerned with the assessment of risks for human health or the environment and involved in communicating and explaining scientific risk issues to the public. Besides following basic rules of

W. Lilienblum (✉)
Hemmingen, Germany

Formerly: Lower Saxony State Authority for Occupational Safety and the Environment, Hannover, Germany

M. Lilienblum
Former: Faculty of Social and Behavioural Sciences, University of Amsterdam, Amsterdam, Netherlands

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good communication, *risk experts* have to find solutions to overcome specific barriers in the dialogue with differently educated protagonists of the public, i.e., laymen, stakeholders, well-informed citizens, other experts, and also with public media. This includes adapting the dialogue to the audience, to explain complex scientific facts and their legal context perspicuously, and to achieve trust and a truthful dialogue atmosphere when discussing with citizens or stakeholders. Eventually, the risk expert should contribute to reach agreements or other options (including disagreements) when discussing risks. For the interaction with public media, the risk expert should keep particular rules in mind. *In conclusion*, awareness of obstacles in communication and acquiring special communication skills and training are mandatory for scientists in the role of risk experts before discussing risks in a public setting.

Institutions involved in risk communication issues, such as many authorities and enterprises, should develop concepts and long-term strategies to build up confidence as an indispensable basis of successful risk communication. Essential prerequisites of such confidence are i) scientific competence and legal independence of the risk experts within the institution (as far as possible by contract) and ii) transparency and a two-way communication between the institution, stakeholders, and the public.

Introduction: Issues and Objectives of Good Risk Communication

Risk communication has been defined as "an interactive process of exchange of information and opinions on risk among risk assessors, risk managers, and other interested parties" (FAO and WHO 2016). Consequently, basic objectives of good risk communication are:

- The *exchange of information* on facts, in particular features, extent, and probabilities of risks discussed
- *Exchange of opinions* about appraisals, concerns, fears, and anxieties about these risks

One major issue in risk communication is that risks, perceived by individuals or groups, are *perceived risks associated with emotions* and corresponding responses. Table 1 provides an overview on some of the factors that influence the perception of risks. Predominantly, these factors are associated with *risk aversion* presumably triggered by spontaneous emotional responses deeply fixed in our behavioral repertoire of survival when the individual is faced to risk or threat (*flight or fight*).

Another major issue of risk communication is the recipient's cognitive bias and consequently misevaluation of risk, often caused by the context of information. Factors favoring cognitive biases are, e.g., the public information flood from different sources or distorted information. In addition, information that is manipulated, overstated, incomprehensible, unfamiliar, ambiguous, or even contradictory raises the concern or the feeling of threat.

Table 1 Factors influencing risk perception

Factor	Increase of perceived risk	Decrease of perceived risk
Perceived catastrophic potential	1000 people affected at the same time	1000 people affected over a greater period of time
Perceived naturalness	Unnatural/human-made hazard	Natural hazard
Perceived controllability	Uncontrollable	Personal controllability
Severity of consequences	Severe consequences (regardless of likelihood of occurrence)	Consequences not severe
Immediacy of consequences	Immediate consequences	Delayed consequences
Affected sub-population	Susceptible groups by age, pregnancy, genetic or acquired susceptibility	None of these groups
Scientific knowledge	Risks unknown to science	Risks known to science
Familiarity of risk	New risk	Familiar risk
Voluntariness of exposure	Involuntary exposure	Choice about exposure
Perceived distribution of risks and benefits	Unequal distribution of risks and benefits	Equal distribution of risks and benefits
Ethical and moral concerns	Risk considered as ethically or morally wrong (e.g., fraudulent acts)	No ethical or moral concerns

From FAO/WHO 2016 (modified)

Ignoring individual emotions or personal values connected to the risk issue would be like talking about the visible part of an iceberg while disregarding the major part below the water surface. With regard to the exchange of opinions, it is often difficult for scientists to deal with the expectations beyond the factual level: Scientists in the role of risk experts usually evaluate risks in a different manner than individuals or public media do (see Table 2). Nonetheless, risk experts may behave similarly to laymen when faced to risks in areas where they are laymen themselves.

Risk management in short is a decision-making process involving considerations of political, social, economic, and technical factors with relevant risk assessment information on a hazard (WHO 2004). Important aims for the *risk management* are mutual understanding in the communication and agreement, e.g., about the implementation of measures for the reduction of risks. Whether such objectives can be achieved, strongly depends on the opinions and interests of the protagonists and stakeholders concerned. The role of the risk expert in such a situation may become difficult in case his opinion is attributed to particular interests; consequently, his objectiveness and credibility may be doubted by individuals or parties with different interests or opinions.

Involved parties in risk communication are as follows:

Risk experts from different institutions (engineers' offices, industry, authorities, scientific institutions)
Citizens with diverse levels of knowledge and education

Table 2 Risk perception and risk appraisal by risk experts and citizens

Risk experts	Citizens
High degree of abstraction	Real and subjective observations
Strict application of scientific methods/ procedures	Preference of intuitive approaches
Application of statistical or probabilistic methods	Expectation of inevitable (deterministic) developments
Determination of acceptable risk values/limit values as basis for measures by risk management	Striving for 100% safety
Comparison of different risk scenarios by mathematical/abstract figures	Consideration of single/separate incidents; refusal of the comparison of different risk scenarios
Statistical average person as a reference, e.g., 70 kg average bodyweight	Personal or social relationship to (potential) victims, sympathy, dismay/shock

Ref.: German Advisory Council on the Environment (SRU), 1999 (modified)

Public media (television, broadcast, print media)
Action groups (citizens), consumer councils
Companies and associations of industry
Other administrative authorities, bodies, agencies
Political lobby groups
Other protagonists or stakeholders

Some of these different parties have in common that, when confronted with warning messages about hazards or risks, they have diverse and often little previous knowledge about the issues. Hence it is important for risk assessing institutions to integrate risk communication as a permanent discourse in their external communication in order to reduce information deficits and to promote a realistic perception of risks in the general population (see ▶ Chap. 58, "Risk Comparison in Toxicology" in this book). Especially government agencies and industrial companies and associations should feel their responsibility to this invitation.

The larger the differences between the groups of the target audience, the more difficult it will be to manage the risk communication process and the higher will be the potential for conflicts. Elements with a conflict potential are:

Previous knowledge of risks and level of information about particular risks
Risk perception
Acceptance of certain risks
Distribution of benefits and expenses of a risk-reduction measure
Extent and kind of the exposure to a risk
Particular interests

Particular interests may be of economic nature including suspected decrease in value (e.g., lower costs for the industry, gain or loss of employment, residential sites close to a planned industrial plant) or of, e.g., political, moral, or ideological interests.

Elements of Effective Risk Communication

Every message is only as credible as the source that tells it! When important decisions have to be taken, the success of the parties depends on their credibility and the trust that other parties have in them. When an obvious charlatan appears more credible than a risk expert, something went wrong in the debate.

Criteria for the trust of citizens in scientific statements are as follows:

Trust in a message
Trust in the person that states it (personal reputation)
Credibility and trust in the information source
Credibility and trust in the scientific institution
Social climate which may ease or constrain the confidence building

Basic Rules of Good Communication

Being proficient in the basics and rules of communication is a requirement for effective risk communication. There is much information easily available in guidebooks and on the Internet about how to achieve and to perform good and efficient communication. It is recommended to join advanced training courses additional to the study of appropriate literature. Taking courses of rhetoric may also be useful but will cover only a subarea.

A message is a basic element of communication. In the following, some background and practical rules are provided on the components of a message: *Every message is only as good as the part of it which is reaching the recipient!* This does not only apply to the factual content but also to three other layers of a message. Based on the psychological work of Paul Watzlawick on communication, ("We talk even when we're not saying anything" is just one of Watzlawick's five axioms on communication), Schulz von Thun (1998) developed the *four-sides model of communication* (also known as *communication square* or *four-ears model*). The four sides have the function to clarify the four layers of a message:

Layer of matters and facts of a message
Self-revealing and *self-disclosure* by the sender
Layer of *relationship* between sender and recipient of the message
Layer of *appeal* from sender to recipient

The simple example of the four sides of the communication square is used just to illustrate that communication is multilayered. Consequently, risk experts would be well advised to look more closely at the basics of communication in order to avoid blunders in communication, to practice one's abilities, and (similarly important) to detect attempts of manipulation by stakeholders.

On the *layer of matters and facts*, the recipient gets information about the communicated issues. Clear structure, logical order of factual arguments, and

comprehensible wording (inclusive explaining of technical terms) can help the recipient to encode the message the way it is intended. The sender's request for feedback about how the message has been perceived can help to detect any misunderstanding and provides the opportunity for correction.

On the *layer of self-revealing and self-disclosure*, the sender gives information about themselves, e.g., about their role in the process of communication, competence, points of view, ideals, other parts of their personality, or about their actual mental state. This partly happens as an intentional self-revealing but also as an unconscious disclosure.

On the *layer of relationship*, the sender communicates openly or subliminally what they think about the recipient or about the way they define the relationship. The way of talking (wording, body language, intonation, etc.) may express, e.g., respect, friendliness, disinterest, or contempt toward the recipient.

On the *layer of appeal*, the sender openly or subliminally attempts to influence the recipient in order to think, feel, or act in the way intended by the sender.

On each of the four levels of a message, each perceived signal triggers a sensation or a reaction of the recipient. On the level of self-disclosure and on the level of relationship, unintended effects of subliminal or unconscious signals of the sender might be particularly problematic.

Risk experts in particular have to take into account that protagonists of other parties, as being recipients, critically compare the factual information with their sensations of the other three levels for incongruities. A message consistent on all of the four levels of communication is one of several important requirements for credibility and trust; another term, which means the same, is authenticity. If all of these four levels of a message reach the receiver and are encoded in the intended way, the risk expert has successfully resolved one of several obstacles with regard to credibility and trust.

Important Requirements for Effective Risk Communication

Additionally to being proficient of the basics of good communication, the risk expert is expected to translate technical terms into a language comprehensible for everybody (including citizens unversed in the risk issue) and to arrange mutual understanding.

Important tools and techniques for this challenging task are as follows:

Translating technical language into everyday language
Simplifying circumstances and line of thought to the essential, without omitting relevant information
Illustrating complex matters with examples and comparisons of everyday life

Useful strategies to gain trust and credibility are as follows:

Disclosure of all relevant factual information and transparency with regard to the expert's personal role/function

Dynamic communication policy, rapid and comprehensive information, e.g., immediately after accidents happened (important for institutions involved)
Reliable adjustment of information
Acknowledging ambiguities and uncertainties
Responding to emotions of the public
Showing presence and leadership skills

Also, the risk expert has to consider that abstract determinism and reductionism, often inherent to a scientific approach, can be rarely reconciled with the thinking of citizens in social relations. Furthermore, good risk communication is not a one-way communication: Sending a message does not mean that the receiver passively absorbs the message like a sponge. It is a two-way communication with both an active sender and active receivers with their own opinions and own perceptions (including contrary positions).

And finally, all parties have to be conscious about the fact that good risk communication is an important requirement, but not a guarantee for comprehension or agreement.

Contact with *News Media* (Press, Television, Broadcast)

Risk experts are in great demand, especially when accidents in the field of chemistry, scandals around food, etc. have happened because of the public attention focused on these issues. However, they are also consulted for less big issues.

There are different reasons why risk experts are getting consulted by the media. It might be pure interest in information, but journalists could also already have a certain idea of how to present a particular issue and which statements (or headlines) ought to be supported by means of an expert. In the latter case, the risk expert would provide a scientific disguise for the story the journalist has in mind; only certain parts of the interview would appear in the article/interview, utilized to support the journalist's idea of the story. Before agreeing to the interview, catch up on the background of the request! Clarify, *in advance*, that you can have a look at the final material for correcting gross errors of reporting before it gets published. However, this request might not always be embraced by the journalist.

Furthermore, the following is recommended:

Preparation
: Think about and prepare *in advance* how you can formulate in a few words what you want to say. It is important to keep it simple so that laypeople can follow it. Also illustrate your statements with examples to make abstract things comprehensible. Besides, illustrations are often better understood, maintained, and reproduced than abstract explanations.

Comprehensibility
: Journalists are busy people and therefore are often only superficially informed. If you leave the translation of your complicated technical wording to the journalist

(because everyday language is necessary for a broad public), the risk of an incorrect reproduction increases. Keep in mind and take care about your statements being clear and comprehensible to laypeople. Also avoid jargon; the meaning of terms has to be (made) clear to non-insiders.

Statements

Time on air or space in articles is restricted: be short and concise. Be aware of the fact that journalists often omit details when editing the material in order to create a clearer picture or to enhance comprehension. Also, the public's ability to maintain information is limited (and reproduction of information even more).

While listening to you, journalists often already listen and think in an editing mode: Which sentences are short and concise; what is an expedient quotation in the context?

Provide the journalist with appropriate (short and clear) statements which are "ready for use"; this increases the probability that the statement will get published correctly (because the journalist can easily use it), and it decreases the risk of an incorrect report due to subsequent need for editing (shortening or simplification).

In certain cases, you can consider a media training session to practice how to appear in front of a camera, how to react on critical or delicate questions, and how to communicate your message effectively in a convincing and intelligible way (*perceived* competence).

Preventive Risk Communication

Basic Rules for Institutions Involved in Risk Management and Risk Communication

Institutions involved in risk management and risk communication should implement a program on good risk communication. Besides the rules and elements described in other sections, some additional rules should be followed to ensure successful risk communication and to achieve building up credibility and trustworthy (EFSA 2017; OECD 2002):

Openness

Openness is crucial to good risk communication and the reputation of an organization. It is important that risk assessments are published in a timely way and that information can be scrutinized how and which decisions have been made. Open dialogue with stakeholders and interested parties is also critical to building trust in the risk assessment process.

Transparency

Transparency is closely linked to openness and is equally important in building up trust and confidence. Transparent decision-making and a transparent approach explaining how an organization works and how it takes its decisions are also crucial.

Preparedness for crisis situations

In the real world, occurrence of fraud, misuse of chemicals, chemical accidents, etc. cannot be excluded. Therefore, institutions involved in risk assessment and risk management need structures and networks for rapid and adequate response in case of crisis situations. As an example, the European Food Safety Authority (EFSA 2011) describes in a guidance how the urgent advice structures are activated, which steps must be followed, and who the actors are and their tasks.

Public Hearings for Preparing Decisions by Authorities or in Politics

The preparation of an official decision, e.g., a public hearing, is a typical case where risk communication is necessary. Due to the legal frame, particular rules should be considered for risk communication. It is necessary to distinguish between public hearings and expert hearings for preparing changes of legislation (laws, ordinances, etc.). The former are based on existing law, e.g., in connection with the approval of hazardous industrial plants, whereas the latter provides more space for different scientific opinions, options, and political debate. It is inherent for legal procedures such as approval that the competent authority is bound by law to approve any submission if the applicant fulfills the legal requirements. In such a case, the authority often has only a small margin of decision, contrary to expectations of citizens concerned. Therefore, it is important for a successful progress of the procedure to provide detailed and clear explanations of the frame of legislation to the public or citizens involved, already at the beginning of the hearing. This may help to restrain unrealistic expectations of citizens with regard to the realization, prevention, or substantial limitations of the project.

A dilemma of risk regulation is that zero exposure of humans to chemical substances is often not possible due to practical reasons or is in conflict with important socioeconomic considerations. Therefore, the concept of acceptable risk has been developed. This concept requires the setting of limits for the exposure to toxic chemicals, i.e., acceptable limit values or other measures that can be considered to have no or a negligible impact on the incidence of adverse effects in an exposed population. Setting the acceptable risk and adequate measures is a risk regulation issue case by case: According to WHO (2004), the acceptability of a risk depends on scientific data, social, economic, and political factors and the perceived benefits arising from exposure to an agent. In complex situations, potential remaining risks (although considered presumably small and in general not measurable) often cannot be excluded and are inherently also part of the accepted risk.

Similarly important is the detailed explanation of basic but often undetermined legal terms by the management of the hearing such as the *principle of proportionality*. This principle is being anchored in the Treaties of the European Union and in the constitutions of the EU Member States and has the function to limit the

extent of restrictions the authority can impose, e.g., because technical measures reducing risks may be expensive. In risk management discussions, the principle of proportionality is often closely connected to the *precautionary principle*. The precautionary principle may be applied in special cases, when scientific uncertainties prevent a full risk or cost-benefit analysis and severe or irreversible adverse effects for a part of the population or for the environment cannot be excluded. For details, the reader is referred to a Communication of the European Commission (COM 2000). In addition, other important legal terms should be explained to the audience such as the *generally recognized codes of practice* or the *current state of scientific knowledge and practice*, if applicable. This kind of hearings is normally managed by a leading official of the competent authority, who is often a lawyer specialized in administrative law. The risk expert should be familiar with such legal terms.

The risk expert in such hearings usually has the role of an appraiser. Often the applicant proposes an accredited or well-respected expert to be appointed by the authority.

The following criteria are essential for generating credibility and acceptance of the appraiser by citizens or stakeholders involved:

Perceived independence
Perceived competence
Consistency of scientific reasoning
Fairness
Willingness and the competence of responding to different opinions or contradictory arguments

Perception of independence is as important as independence itself, but it is subjective. Independence and zero tolerance of conflicts of interest (CoI) are driven by societal organizations. An important criterion of independence is the affiliation of the risk expert. Risk experts from industry are normally not regarded as independent although they often strive to be. A consultant or appraiser, dependent on mandates from industry because of a high degree of specialization, may be generally regarded as independent; however, he should preferably demonstrate his independence and credibility by consistency of reasoning and persuasiveness. Competent authorities and other public scientific institutions often have introduced a passage in their rules of internal procedure which assures that the scientist is independent from instructions with regard to scientific appraisal issues he is responsible for. Usually this does not apply to risk managing authorities such as ministries where often hierarchy and political considerations predominate. In case the risk scientist acts the role of an appraiser in public hearings or at other public occasions, he should explain his role in the procedure as detailed as necessary and also comment on the issue of his scientific independence, if applicable.

Public hearings intended for the preparation of regulatory changes may leave more scope for discursive procedures and external moderation. Also for this kind of hearings, the above-described role of the risk expert applies.

Risk Communication and Training Preventing Hazardous Incidents and Transport Accidents with Release of Dangerous Substances

The life cycle of many risk issues can be divided into four phases: In the *latency phase*, the risk issue is not yet a matter of public discussion but only known to insiders. In the *emergency phase*, slowly or fast, the risk issue arises in public discussions and media and may develop into a "problem." In case of a critical event such as a plant accident with release of dangerous substances, the emergency phase is very short and the problem may develop toward a *crisis.* In the subsequent *regulation phase,* the risk issue becomes the matter of regulatory actions (Wiedemann et al. 2011). Therefore, all organizational and communicative actions are primarily focused on warding off any damage or on limiting the extent of damage. However, good risk communication starts far in advance to hazardous incidents, namely, by specifying procedures for approval/authorization of industrial plants with intrinsic dangerous potentials. Required information and communication includes scenarios of accidents, emergency (management) plans, and appropriate practice and exercises on a regular basis (SFK 1997; OECD 2002; Wiedemann et al. 2011).

By information and training in advance, the emergency staff is usually familiar with the local conditions and also with possible accidental releases and will arrive immediately in case of an incident.

With regard to the transport of hazardous goods on roads, rails, or shipping lanes, the focal point is concentrated on safety-related provisions on the means of transportation in order to prevent accidents. This applies also to the transport company's reliability, aiming to reduce the probability of technical or human failure and to limit the impact of an accident on human health or the environment, respectively.

If hazardous incidents happen, a smooth and rapid interaction with adequate communication lines between the responsible institutions and authorities is crucial for the conduct of emergency plans, in particular when substance release exceeded the plant boundary. This requires careful planning and coordination in order to limit damage to a minimum.

In addition to the elucidation of the cause(s) of an accident, extensive information of the public on hazards or risks, which may have existed or may still exist, has to be carried out. The necessity of medical diagnostic examination or treatment of exposed individuals has to be decided case by case. Such decisions require adequate scientific justification and knowledge of the adequate chemical and medical investigation methods available but also some (political) flair in order to avoid public criticism.

Risk or Hazard Information on Chemical Substances and Products

Last but not least, an important area of preventive risk communication is the design of written product information for consumers and for workers handling hazardous materials. Form and extent of such product information is partly stipulated by laws,

by regulations, or by nonlegislative technical rules and standards intended as technical minimum requirements.

Some typical examples of important product information (see also ▶ Chap. 68, "Health Hazard Classification and Labelling" Author Desel of this book) are as follows:

Labeling of dangerous substances, preparations, articles with regard to their dangerous properties by hazard pictograms, hazard statements (risk phrases), precautionary statements, and safety data sheets (SDS) according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS)
Labeling of ingredients, e.g., additives in food, often listed as codes (E numbers) or the list of ingredients in cosmetic products
Instructions for use and warning notes of medicinal products or articles of daily use

Beyond the official legally provided requirements, the written information has to be short but complete and comprehensible. Editorial review by laypersons is recommended. Especially in case of complex information, graphic images may be more comprehensible than pure text. Readability (the fine print!) is often more important than the handiness of the package insert; not everybody can be expected to have as good eyes as the designer of the leaflet.

Cross-References

▶ Health Hazard Classification and Labelling
▶ Risk Comparison in Toxicology

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Dealing with Diseases That Have Been Attributed to Chemical Exposures

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Thomas Zilker

Contents

T. Zilker (✉)
Formerly Department for Clinical, Toxicology, Medizinische Klinik, Klinikum rechts der Isar, Technical University of Munich, Munich, Germany
e-mail: Thomas@Zilker.de

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Abstract

The cause behind the so-called MCS or IEI (Idiopathic Environmental Intolerance) is a mental or psychogenic or psychosomatic disorder.

Although the regulated threshold values in environmental media are aimed at virtually eliminating adverse health effects due to toxic substances, there are patients who attribute their illnesses to pollution. Only rarely is it possible to construct causality between the symptoms complained of and an exposure to toxicants. The communication between the physician and the patient is often disrupted. Only close cooperation between the family doctor and the doctors of environmental medicine can alleviate the suffering of these patients.

Introduction

Patients presenting themselves to the doctor as being environmentally ill often complain of a strong feeling of being unwell, the cause of which had not been medically explained so far. The symptoms can include listlessness, fatigue, disturbed concentration, muscle pain, and irritation of mucous membranes. Patients attribute the cause to pollution and/or an increased sensitivity to ubiquitous pollutants. They hope for identification of the contaminant, either by having the specific symptoms pertaining to that contaminant or by means of pollutant analysis (Staudenmayer et al. 2003a, b; Bornschein et al. 2006). This vague clinical picture is also referred to as *multiple chemical sensitivity* (MCS). This concept is based on the notion that sensitization to various chemicals is triggered by previous exposure to pollutants, as a rule usually accompanied by odor intolerance.

Regulatory aspects are present in two ways. Firstly, patients demand more stringent threshold values so that they, with their hypersensitivity, are protected. Secondly, with its human biomonitoring, environmental medicine has a method of investigation, the results of which can be assessed on the basis of scientifically derived values (reference values, HBM values).

Environmental Health Investigation

The examination of the environmental health patient consists, as is common, of taking a medical history, a physical exam, and – if necessary and useful – apparatus-based diagnostics and laboratory tests. Consultations with specialists should be requested if the symptoms are outside the specific area of expertise of the examining doctor. The following disciplines are required: internal medicine, clinical toxicologist, dermatologist, allergist, neurologist, occupational medicine, laboratory medicine, and – last but not least – the psychosomatic specialist and/or

psychiatrist. When it concerns prevention and counseling, Public Health specialists and doctors from the Public Health Service are involved.

Medical History

While this statement reportedly made by a wise physician certainly still holds true: "Whoever does not have a diagnosis after taking the medical history, is badly off," apparatus- or laboratory-based examinations are often given precedence. But as experience has shown, patients with ailments associated with environmental health issues are often not helped by these kinds of diagnostic investigations, and so taking the medical history is of utmost importance. This serves not so much the purpose of inquisitorial questioning of the patient, but rather of creating a ***basis of trust*** from the very beginning. Although time consuming, the patient should be allowed to express himself and should be listened to.

However, in order not to miss or forget important symptoms or associations, giving patients a ***questionnaire*** to answer beforehand has proved invaluable. As these questionnaires cover all possible symptoms and associations imaginable connected to living and working areas and are therefore very extensive, it makes sense to let the patient have a questionnaire well in advance of the consultation and to give oneself enough time to study it before seeing the patient.

The following categories are covered in the questionnaire:

1. Symptoms, with information about the time of onset, the duration, intensity, and the frequency
2. The disposition of the subject regarding familial clustering of diseases, hypersensitivities, allergies, and certain diseases
3. Potential exposure due to lifestyle factors such as natural stimulants, smoking, drugs, medication, sport, and leisure activities

Furthermore, ***exposure possibilities*** within the living accommodation, the surroundings, the household furniture, the use of domestic chemicals, and potential exposure in the workplace, by means of traffic, food, animal contact, and travel have all to be elicited.

Should suspicion fall and harden on a particular exposure, the search for possible vectors must be carried out. To this end, air, water, dust, food, utensils, products, and clothing are called into question. Of course, the maximum concentration that can be present in these vectors also plays a significant role. It is important to ask about first-time exposure and a one-off acute event. The doctor taking the history should not get too set in any one direction, but should remain open. He has to accept the patient's explanations, but must not overlook any symptom complex that might indicate a non-environment-related disease. That is why a doctor well trained in internal medicine with a broad subject knowledge and who has possibly come across many, even rare, diseases is predestined to be especially suitable for taking patient case histories.

Uncommon Somatic Disorders

In our department, for example, some uncommon diseases have been diagnosed in these patients, such as acromegaly, hyperthyroidism, hypothyroidism, Wilson's disease, Mediterranean fever, cardiomyopathy, diabetes mellitus, insulinoma, pheochromocytoma, chronic lymphocytic leukemia, sarcoidosis, and various connective tissue diseases. In the neurological department, we came across Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, and meningioma with disturbance of the sense of smell. Chronic carbon monoxide poisoning accompanied by nausea and headache, usually due to faulty flues and chimneys, are among these misattributions. Uncovering medical or neurological disorders despite the aforementioned diagnoses is still somewhat rare.

Disorders Not Able to Be Diagnosed by Conventional Medicine

As a rule, none of the somatic disorders defined by conventional medicine are diagnosed. In the case of somatic disorders, when the medical history is being taken, very often quite specific indicators are mentioned, for example, pain at very typical locations such as the stomach, gall bladder, kidneys, lower abdomen, or thorax, after meals, during exercise, climbing stairs, in the night, or a loss of appetite with weight loss, bloody stools, blood in the urine; however, patients with environmental disorders do not present in the same way. Rather, they complain of pain that cannot be exactly localized, and their presenting signs are vague but manifold. Such patients specify muscular pain that migrates and is not precisely localized. Reports of occurrences of feelings of numbness without exact localization and constancy arise repeatedly. Problems such as indigestion with nausea but without vomiting or increased stool frequency are also often specified. Nervousness, fatigue, dizziness, a burning feeling on the skin or mucous membranes, palpitations, dyspnea without cyanosis, or spasticity are also complained of frequently. Disturbed sleep patterns are also included, but without specifying whether the problem lies in falling asleep or sleeping through the night.

Social History

Besides taking the medical history and asking about all the presenting signs and symptoms, care should be taken to enquire after the social environment. It is to be noted that most of the patients appear dissatisfied with their social environment, but not because money is short, or the employer is putting on the pressure, or the partner is being difficult, but rather because they are experiencing problems due to their symptoms that were there in the first place.

Previous Findings

Before patients visit a clinic for environmental medicine, as a rule they have already been seen by doctors from various disciplines. Many of these patients keep a special file folder that they bring with them to the consultation, usually containing files of numerous previous examinations. Mostly, the previous examiners could not decide on a common diagnosis. Such patients lay great importance on receiving original copies of the findings as quickly as possible in order to store them in this folder. Included in these investigations, besides laboratory screening and human biomonitoring tests, there are investigations of their surroundings. These had usually been carried out by institutions specialized in measuring minute concentrations of chemicals in the air, in dust, and in materials to be found in the home environment. Upon exceeding the background values or reference values of one method, a whole host of complaints are then specified that could match the chemical that has exceeded the reference value.

Findings

A complete physical examination, which should have an internal medicine focus as well as a neurological focus, is essential! One works from top to bottom. In addition to the reflex test, the neurological exam incorporates the extended-hand test, Romberg's test, Babinski, Hackengang, evaluation of superficial and deep sensibility, and testing the diadochokinesis.

Blood Sampling

At a time of cost constraints within health care, some restrictions on laboratory tests should be in place. They should be selective and targeted at searching out any organ pathologies that may already have been indicated in the examination. As a rule, electrolytes, serum liver values, serum kidney values, blood sugar, and blood count with leukocytes, erythrocytes, and platelets are determined, along with an inflammatory parameter, preferably CRP. In respect of the biomonitoring, according to laboratory guidelines either blood or urine should be collected, depending on the pollutant. The urine should be checked at the same time for bacteria and erythrocytes.

Monitoring in Environmental Medicine

The patients' hypothesis is that a pollutant has caused their suffering. Case history and chronological sequence of the complaints should indicate the direction the search has to take to find the source. Basically, one can distinguish between

environmental monitoring (ambient (bio)monitoring) and human biomonitoring. Environmental monitoring, in connection with patients, only makes sense if a suspected source is known. Often, this is the dwelling place. A possible increase in pollution in the dwelling place can be ascertained by measuring the ambient air, preferably preceded by a specialized tour of the dwelling and history taking with respect to any potential sources of contamination. Nowadays, only rarely and usually only temporarily following any new fittings or renovations, can an increased concentration of pollutants be found within the dwelling that are likely to induce ailments. In this connection, odor nuisance can be a relevant factor.

Human biomonitoring can be divided into exposure biomonitoring, effect biomonitoring, and susceptibility biomonitoring. With ***exposure biomonitoring***, the substances absorbed from the environment are registered in body materials, mostly in blood/plasma/serum and/or urine. Biomonitoring of hair, saliva, breast milk, or fatty tissue rarely makes sense. It is not correct to determine formaldehyde contamination on the basis of the identification of formic acid in urine, because formic acid accumulates during metabolism even without exposure to formaldehyde. One speaks of ***effect biomonitoring*** if the poison itself can be measured less well than one of its effects, which thereby do not have to be toxic yet. A classic example of effect biomonitoring is determining plasma cholinesterase (PChE) to assess a contamination with organophosphates (also see ▶ Chap. 42, "Assessment of Background Exposure and Additional Exposure by Human Biomonitoring"). ***Susceptibility monitoring*** is served by measuring biomarkers that indicate the individual susceptibility to toxic influences. An example of this is determining the expression of detoxification enzymes, or, in the field of allergology, the determination of immunoglobulin E.

Specimen Collection and Processing Procedure

The first thing to consider is what the appropriate materials to use for exposure biomonitoring are. As a rule, blood is a suitable medium to determine an exposure occurring within the near past, while urine covers a somewhat longer time period and especially because most substances are found in higher concentrations in urine than in blood. For some substances, hair can provide evidence of an exposure that took place up to several months previously. However, in the case of hair, it is difficult to distinguish between internal and external exposure. Blood fat provides a suitable medium to determine lipophilic substances. For this purpose, larger amounts of blood are needed. Teeth or bones allow for gauging exposures lying further in the past, even over the course of years.

In order to choose the most suitable material for biomonitoring, it is important to know about the metabolism of a particular substance. So, for material with low renal excretion and high metabolism in the liver, blood/plasma/serum are better and more suitable for analysis than urine. The insecticide llindane is a classic example of this. Unaltered lindane appears in urine in only the slightest amounts, making plasma the more suitable material for ascertaining lindane.

As a rule, 10 ml of EDTA blood and/or 50 ml of urine are required. It is important to ensure that no contamination occurs. As a matter of principle, venous blood, for example, should not be drawn via metal cannulae when ascertaining levels of metals, but rather by means of polyethylene cannulae in situ. This is particularly critical when drawing blood for aluminum, chromium, and nickel analysis, because for these metals there is a high risk of contamination from the surroundings. Urine samples are collected as a random specimen or timed collection specimen. In turn, the most suitable collection vessels for this purpose are made of polyethylene or polystyrene. For determining organochlorine biocides (e.g., DDE, HCB, PCB) in blood, glass tubes should be used throughout the procedure.

Evaluation of Results of Human Biomonitoring

The assessment of the results can be done in two ways. On the one hand, in comparison to the reference value that reflects the background level to be found nowadays in the population, and on the other hand, by means of toxicologically derived values such as the BAT values for the workplace or the HBM values for the environmental area (see "Cross-References").

Reference Values for Human Biomonitoring

The comparison of the measured level of contamination of the patient with the reference value provides information about whether a greater than average concentration is present. Excess above the reference value has per se no toxicological relevance.

Toxicologically Derived Human Biomonitoring Values

A comparison of the measured level of contamination of the patient with the toxicologically derived HBM value provides information about the degree of health risk. Unfortunately, HBM values are only available for a few substances.

Limitation of Biomonitoring

Many patients who visit an environmental medical clinic are not aware of which pollutant is causing their ailments. This can lead to an ineffectual, very broadly based biomonitoring. From this, one might conclude that biomonitoring does not have a good cost-benefit ratio at a time when savings are called for in health care. This contrasts with legal concerns: If a patient feels that a certain substance is poisoning him, he has the right to demand clarity. A common problem lies in the interpretation of results. Doctors often certify that, when a reference value is exceeded, then a

patient has a health-related risk. This is pure nonsense, because the reference value is not derived toxicologically. Announcing such erroneous findings results in patients being frequently misdiagnosed as "poisoned." If everything is unclear, further help can be obtained via ambient monitoring. If under ambient monitoring increased exposure to a specific toxicant is established, under certain circumstances this can be specifically searched for by human biomonitoring. Ambient biomonitoring is, however, not a medical service and, as a rule, must be paid for by the patients themselves.

Dealing with Environmental Patients from a Psychological and Psychiatric Perspective

With the present state of knowledge, one must assume that a psychological component (toxicophobia, nocebo effect, phantom risk) plays a role with many patients. In order to meet patients' needs, in addition to the somatic-orientated and well-trained doctors such as general practitioners or internists, there should be doctors trained in psychosomatic medicine or psychiatry.

Psychiatric Diagnostics

Before the therapy, the "gods" have made the diagnosis. As already pointed out above, all known serious somatic disorders must be excluded. Once this is done, the patient has to be examined by a psychiatrist/psychosomatic doctor. As a large-scale study done by us on 308 patients in cooperation with the toxicology and psychiatry departments has shown, in 35.3% of them the presenting symptoms can be completely explained as belonging to a mental disorder. In 21.6%, there was an underlying physical condition that could explain the symptoms adequately. In 22.2%, the simultaneous presence of a mental disorder and a somatic disorder could justify the symptoms. In 1.6%, the patients' ailments could be explained by the impact of a pollutant. This leaves a group of 14.1% remaining where the ailments could neither be explained somatically nor mentally. These patients were suffering from an impairment of well-being that was neither mental nor psychosomatic.

For an experienced psychiatrist or doctor of psychosomatic medicine, it should not normally be difficult to narrow down the type of mental dysfunction, so long as the patient is prepared to undergo one or more verbal consultations. However, if one wants as scientific and comprehensible a diagnosis as possible, a battery of psychological and/or psychiatric tests is essential. In our study of over 300 patients, of course, not all environmental patients were found to have the same personality or psychiatric disorder, but rather virtually any kind of distinctive personality type or known psychiatric diagnose could be found (Fig. 1). In these studies, we used as test batteries the Symptom Check-List (SCL-90) which is measuring the recent psychological stress and the Structured Clinical Interview following the Diagnostic and Statistical Manual of Mental Disorders (SCID-DSM-IV) Axe I and Axe II.

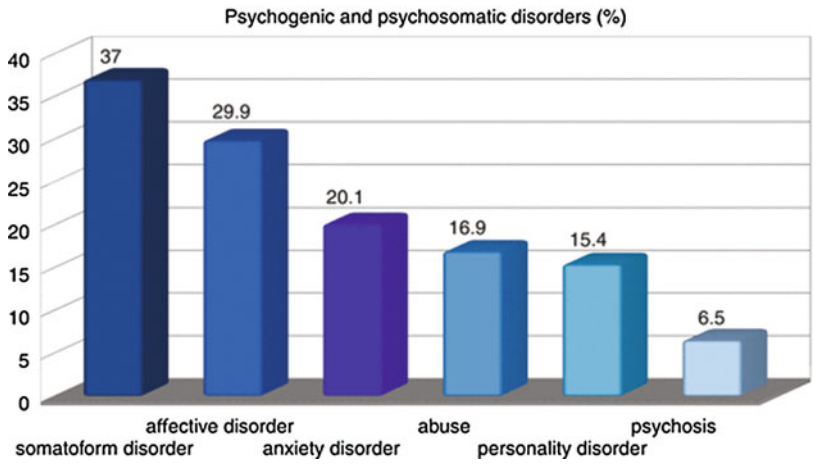

Fig. 1 Psychiatric diagnoses in patients with environmental disorders ($n = 308$) (some of the patients have multiple diagnoses)

What they all have in common is simply the belief that the perceived ailments are the result of environmental pollutants. Under somatoform disorders, we understand psychosomatic diseases; under affective disorder, depression and/or manic depression; under anxiety disorders, the phobias; and under psychotic disorders, the schizophrenias.

Others like Skovbjerg et al. (2012) from Denmark used a revised SCL-test the SCL-92 and compared the found symptoms with a healthy control group. Their results confirmed a positive association between symptoms of depression and symptoms of IEI-patients. These depressive patients exhibited significant more CNS and mucosal symptoms. A further association was given between the symptoms and the lack of social support whereas recent severe life events did not influence the symptoms. Nearly 70% of the patients belong to a low occupational social class.

Further Models of the Origin of IEI

An intensive literature review by Viziano et al. (2018) from Italy including 34 studies with the emphasis on sensory perception concluded that in IEI-patients a multisensory perception disruption is prevailing. In a study by Chiaravalloti et al. (2015) comparing IEI-patients with healthy controls by (18) F-FDG PET/CT the cortical activity in IEI-patients differed from that in healthy individuals. Micarelli et al. (2016) found in IEI-patients a defective central and peripheral vestibular processing by a battery of otoneurological tests. In a study by Andersson et al. (2009), patients with IEI were compared with healthy subjects using a chemosomatosensory, olfactory, and auditory

event-related potentials. The IEI subjects did not get habituated to the stimuli. The results indicate an attention bias, an enhanced sensitization, and a different cognitive response to chemical exposure.

Therapy

Treatment is determined by the diagnosis, of course. If indeed a relevant pollutant exposure can be proved, then the avoidance of this exposure is the decisive therapy. However, if a pollutant exposure is only suspected on the basis of a psychiatric disorder, then psychotherapy comes into play. In the case of ***anxiety disorders***, behavior therapy brings fast and good results. In a few of these anxiety disorders, treatment can also be supported by use of psychotropic drugs. The ***depressive*** disorders usually require a combination of psychotherapy and antidepressant pharmacotherapy. Depending on the severity of the depression, treatment with antidepressants can stand in the foreground (major depression), or a balanced combination therapy consisting of medication and psychotherapy as in the case of milder forms of depression (dysthymia).

Substance abuse necessitates detoxification and then withdrawal treatment in an appropriate, specialized clinic that works particularly with group therapy and sociotherapy. Following on from this, self-help groups are particularly meaningful. Schizophrenic disorders require treatment with predominantly high-potency neuroleptic drugs and social therapeutic measures after resolution of the productive symptoms. ***Personality disorders*** are unfortunately difficult to treat. Sociotherapy, psychotherapy (dialectic behavioral therapy DBT; Transference focused therapy TFP), and psychotropic drug therapy can lead to improvement.

There is repeated emphasis on not taking patients with environmentally associated disorders seriously, and that they should not be unnecessarily ***psychiatrically treated***. Which normal-thinking, emphatic-feeling doctor would not take such a patient seriously? On the other hand, psychiatry is part of conventional medicine; "psychiatrization" is not done by doctors. Rather, this is more a phenomenon of society, and it is actually time to overcome stigmatization by means of education. To this end, long years of positive public relations are needed, as has long been the case for other kinds of impairment. Of course, it is useless to try to talk the patient out of his notion that his symptoms are related to environmental toxicants. Letting go of these symptoms is only possible at the end of treatment. Especially in the case of somatic disorders, symptom-orientated psychotherapeutic measures seem sensible. One begins with relaxation training, such as autogenic training, progressive muscle relaxation, or hypnosis. This is combined with deep psychologically orientated focal therapy and cognitive behavioral therapy. The aim is to reduce social isolation and symptoms.

Mechanisms need to be found to help other things become more meaningful rather than just ***dealing with the toxicant*** (coping strategies). This way, quality of life is improved. Forces, that is healthy aspects of the psyche, are reactivated. In individual cases, there are reports of success in the aforementioned therapy forms

with these kinds of environmentally related somatic disorders. Patients that exclude any psychogenesis – and they are many – cannot be reached at this point in time. Patients who are ambivalent about accepting a psychosomatic illness are treatable with patience. Patients who accept psychotherapy are to a large extent capable of improvement and in about 40% (personal experience) of cases can be cured.

It is interesting that even though patients seek help from alternative medicine therapy forms, they mostly do not break off contact with conventional medicine and carry on expecting help from that quarter, too. This may be an indication that alternative therapies such as chelation agent therapy, antioxidant therapy, electro-acupuncture, or cleansing methods by means of hydrotherapy or Ayurveda therapy do not always lead to success. The psyche needs a valve in order to stabilize itself. Only if changes are possible can the symptoms be minimized. Suppressed conflicts or guilt is often the reason for externalizing, which means that the blame (the toxicant) for a particular plight is sought for in the outside world, because otherwise the burden of internal tensions would be unbearable. It is my wish that such patients may be helped, but this will only work with interdisciplinary cooperation and patience.

Cross-References

- ► Assessment of Background Exposure and Additional Exposure by Human Biomonitoring
- ► Importance of Exposure Level for Toxicological Risk Assessment
- ► Limit Values and Guideline Values in Regulatory Toxicology

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REACH and CLP: Its Role in Regulatory Toxicology

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Walter Aulmann, Miriam Große Hovest, Davina Lemm, and Nathan Pechacek

Contents

Abstract

This chapter intends to illustrate the general framework of the European Chemical Regulation "REACH" (Registration, Evaluation, Authorization, and Restriction of Chemicals). Special focus will be on the aspects which are important for a regulatory toxicologist. Objectives, scope, and basic rules are explained. The most famous REACH principle is "no data – no market." So-called "Klimisch reliability criteria" are decisive to determine whether data are suitable for a hazard assessment under REACH. Non-animal-testing approaches such as in vitro methods or weight-of-evidence approaches are encouraged by the regulation.

W. Aulmann (✉) · M. Große Hovest · D. Lemm
Ecolab Deutschland GmbH, Monheim, Germany
e-mail: walter.aulmann@ecolab.com; Miriam.GrosseHovest@ecolab.com; Davina.Lemm@ecolab.com

N. Pechacek
Ecolab Inc, Eagan, MN, USA
e-mail: nathan.pechacek@ecolab.com

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Registration entails broad-scale derivation of no effect levels for Man and the environment. Exposure scenarios serve as standardized communication tools along the supply chain to convey information on chemical safety under intended use.

Since its implementation more than 10 years ago REACH continued to adjust dynamically to new developments in regulatory toxicology. Several new alternative methods became part of the registration package. They aim at reducing, refining, and replacing animal tests. However, a reverse trend can be observed as well: a reduced acceptance of weight-of-evidence assessments by evaluating authorities. This could lead to even more animal tests in the future for hazard identification.

The 2006 European REACH regulation set a precedence for the world. It established a standard which is increasingly taken as a role model for the chemical regulation in other countries and economic areas.

Keywords

European Regulation · ECHA · Registration · Evaluation · Authorization · Restriction · DNEL · exposure scenarios · Klimisch criteria · Weight-of-evidence approach

Abbreviations

BfR	Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung)
C&L	Classification and labeling
CMR	carcinogenic, mutagenic, reproductive toxicant
CSR	Chemical Safety Report
DNEL	Derived No-Effect Level
DU	Downstream user
ECHA	European Chemicals Agency
ES	Exposure Scenario
eSDS	Extended Safety Data Sheet
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
GLP	Good Laboratory Practice
IUCLID	International Uniform Chemical Information Database
NOAEL	No Observed Adverse Effect Level
PBT	Persistent, Bioaccumulative, Toxic
PCC	Poison Control Centers
POD	point-of-departure
RMM	Risk Management Measures
SEA	Socio-Economic Analysis
SIEF	Substance Information Exchange Forum
SME	Small or Medium Enterprise
SVHC	Substances of Very High Concern
vPvB	very Persistent very Bioaccumulative

Introduction

In its 1999 "White paper" the European Union published the result of an analysis of the existing chemical legislation identifying several problems. The paper challenged the existing allocation of resources applying different approaches for new and existing substances. For new substances, even at low tonnage levels, a burdensome and expensive notification was required. On the other hand, producers were not obliged to submit similar data packages for existing substances. Moreover, the systematic evaluation of existing chemicals had turned out inefficient.

After an intensive dialogue with all stakeholders, the "REACH" regulation was adopted in 2006. The acronym "REACH" stands for *R*egistration, *E*valuation, *A*uthorization, and Restriction of *Ch*emicals (European Union 2006a).

Objectives, Scope, and Implementation of REACH

REACH aims to ensure a high level of protection of human health and the environment, while at the same time enhancing competitiveness and innovation with the focus on less hazardous materials. The regulation applies to substances on their own, substances in mixtures and in articles and is directly applicable in all member states of the European Union.

For the management of the technical, scientific, and administrative aspects of REACH at the Community level, a new institution was founded. This is the European Chemicals Agency (ECHA) in Helsinki, Finland. In order to support industry and authorities to meet their obligations under REACH, technical guidance documents were developed involving experts from various stakeholders (e.g., industry, member states and non-governmental organizations). These documents aim to facilitate the implementation of REACH. They are not prescriptive, but summarize generally acknowledged good practice (ECHA 2013–17). It should be kept in mind that these guidance documents are not legally binding.

In order to maintain the workability of the system, reduced requirements from the obligation to register exist for intermediates under strictly controlled conditions, as well as some exemptions for selected groups of substances (e.g., polymers). Compared to the previous system, an important change is the shift in responsibility for the risk management of substances, which now resides with the industry. Depending on its role as a manufacturer, importer or downstream user (e.g., formulator) industry must comply with specific duties and obligations. A major task for industry is to provide data by filing registrations to ECHA.

At this point in time it is noteworthy that REACH was not the only major change in the regulatory landscape. At about the same time the Globally Harmonized System (GHS) was introduced in the European Union with the adoption of the "CLP-Regulation" (European Union 2008). The regulation sets the ground for classification, labeling, and packaging in the European Union. The idea of GHS goes back to the United Nations Conferences on Environment and Development in

1992 and 2002 (Johannesburg Earth Summit) which decided to develop globally harmonized criteria for classification and labeling.

There are many close links between CLP and REACH. Both regulations make use of a uniform terminology and definitions in order to ensure maximum consistency in the application of chemical legislation within the European Union in the context of global trade. The infrastructure used is basically the same. Both regulations are steered by the same legislative body, the European Chemicals Agency (ECHA). National helpdesks have been established to provide advice to suppliers and any other interested parties, particularly small and medium sized enterprises (SMEs), on their respective responsibilities and obligations under both regulations. The same applies to the technical guidance document for the application of the CLP criteria. It is included in the compendium of supporting documents for REACH on the ECHA website.

Registration of Chemical Substances: "No Data – No Market"

The review programs on existing chemicals had indicated that safety data were often lacking even for high production volume chemicals. To overcome this issue, REACH obliges manufacturers and importers of chemicals to systematically compile safety information in a registration dossier.

Tonnage per year manufactured or imported serves as a crude indicator for exposure of Man and the environment, which triggers the extent of information that must be filed in the registration.

Information Gathering and Closing of Knowledge Gaps

A registrant who manufactures or imports a substance must collect all available and relevant information for hazard identification and assessment. In many cases, the information gathered consists of test data. However, other types of information such as QSAR and Read-Across may also be accepted, especially when used in a Weight-of-Evidence (WoE) approach (see below). This means that registrants use combined evidence from multiple sources to assess a substance property. In certain circumstances, generating standard data required by REACH may not be necessary (e.g., no exposure to humans and the environment throughout the life cycle of the chemical) or technically not possible. For such cases, REACH provides the option not to provide (i.e., waive) such information.

The registrant is obliged to incorporate all relevant available information in the registration dossier, using IUCLID, a software provided by the European Chemical Agency. To that end the existing physico-chemical, toxicological, and eco-toxicological information are gathered.

Sharing of information on substances among the registrants is strongly encouraged by REACH to reduce testing on vertebrate animals. Information must be shared

with other registrants in a substance information exchange forum (SIEF). The task of a SIEF also includes to agree on a classification.

REACH came into force in 2007. So-called phase-in substances ("existing substances") benefited from a 10-year transition period with extended registration deadlines. Deadlines set for registration were tonnage- and hazard-driven. Substances with the highest production volumes, classified as CMR (carcinogenic, mutagenic, or toxic to reproduction) or toxic to the environment, needed to be registered first. The registration deadline for the lowest tonnage band of phase-in substances ended in 2018 (see Fig. 1).

For all substances registered after the 2018 deadline for non-phase-in substances, a so-called "inquiry" has to be sent to ECHA to make sure that the substance intended for a new registration was not filed in parallel by another actor. ECHA must approve the inquiry confirming that to their knowledge no duplicate registration efforts are undertaken for the described chemical. Without this confirmation testing on vertebrates in order to meet the information requirements is prohibited.

In case of data gaps, the registrants must generate new data for all substances to be registered or provide justification on why the data is not needed. For example, certain tests can be waived based on physico-chemical properties or on exposure.

Also, information on exposure, use, and risk management measures must be collected.

For the tonnage up to 100 t/year, the information requirements are laid down in annexes VII and VIII (see Table 1).

If relevant information for more endpoints is available, this needs to be submitted as well, regardless of whether information on this given endpoint is required at this tonnage level or not.

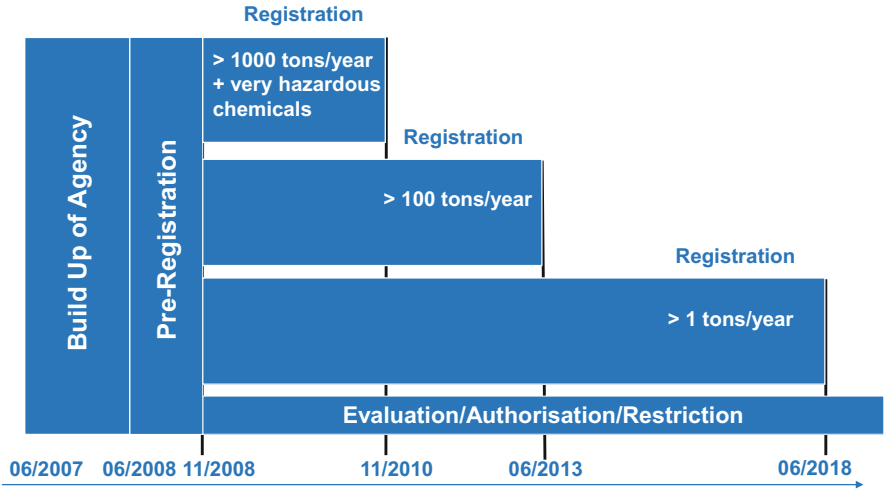

Fig. 1 Timeline for the registration of phase-in substances

Table 1 Information requirements for substances below 100 t/year

Annex no	Endpoint
VII	Melting/freezing point
VII	Boiling point
VII	Relative density
VII	Vapor pressure
VII	Surface tension
VII	Water solubility
VII	Partition coefficient n-octanol/water
VII	Flash point
VII	Flammability
VII	Explosive properties
VII	Self-ignition temperature
VII	Oxidizing properties
VII	Granulometry
VII	In vitro skin corrosion
VII	In vitro skin irritation
VIII	In vivo skin irritation/corrosion[a]
VII	In vitro eye irritation
VIII	In vivo eye damage/irritation[a]
VII	Skin sensitization[b]
VII	In vitro gene mutation in bacteria
VIII	In vitro cytogenicity study in mammalian cells or in vitro micronucleus study
VIII	In vitro gene mutation in mammalian cells
VII	Acute toxicity: Oral
VIII	Acute toxicity inhalation
VIII	Acute toxicity: Dermal
VIII	Short-term repeated dose toxicity (28 day)
VIII	Screening for reproductive/developmental toxicity
VII	Short-term toxicity on invertebrates (Daphnia, acute)
VII	Growth inhibition study aquatic plants (algae)
VIII	Short-term toxicity on fish (fish, acute)
VIII	Activated sludge respiration inhibition test
VII	Ready biodegradability study
VIII	Hydrolysis as a function of pH
VIII	Adsorption/desorption screening

VII – requirements of annex VII – 1 to <10t/y
VIII – requirements of annex VIII – 10 – < 100 t/y
[a]In vivo study shall only be considered if in vitro study methods are not applicable or results obtained are not adequate for classification and risk assessment
[b]Initially performed in vitro/in chemico – in vivo testing if in vitro tests methods are not applicable or results obtained are not adequate for classification and risk assessment

Compared to the requirements for the tonnage bands up to 100 t, the test program for *higher* tonnage levels needs to be tailored in a substance specific manner.

Substance characteristics and information already gathered will influence the data requirements for the next stage (i.e., under annex IX and X). The following additional toxicological information may be required under the regime of annex IX and X:

- Subchronic toxicity study (90-day)
- Prenatal developmental toxicity study
- Extended One-Generation Reproductive Toxicity Study (EOGRT)
- Carcinogenicity study
- In the field of ecotoxicity and environmental fate, further investigations may be needed on effects on aquatic organisms after long term exposure (invertebrates and fish)
- Toxicity on terrestrial organisms
- Long-term or reproductive toxicity to birds
- Bioaccumulation
- Degradation

At this tonnage level the registrant must file a test proposal upfront. The proposal needs to be endorsed before the registrant can launch any test (see also Table 5).

In order to generate data on intrinsic properties of substances for a registration under REACH, the test method regulation 440/2008/EC must be consulted (European Union 2008–2017). It contains a compendium of methods for the determination of physico-chemical properties, of toxicity and other health effects, and of ecotoxicity. The regulation is regularly updated and accounts for progress that is being made in the OECD program for the development of test methods (OECD 2005). Due to different processing speeds in incorporating new methods into the regulation, the OECD and EU compendium do not always match each other. In some cases, the OECD was ahead such as in tests for eye damage (OECD 437 and 438). In other cases, the EU compendium was ahead, such as a test for skin irritation (Test method no B46).

REACH considered the tremendous progress in the last 10 years in the development and application of in vitro methods. In the case of investigating local skin and eye effects, hazard classification is often possible in the absence of in vivo tests. For example, until 2009 animal-free assays existed only for the identification of skin corrosion; however, this changed with the acceptance of a test method using reconstructed human epidermis for the investigation of skin irritation (ECHA 2017a). Unless the substance does ***not*** fall under the applicability domain of the test, the full scale of hazard categories can be addressed now without the use of animal tests, be it skin corrosion, skin irritation, or no classification (see also flow chart – Table 3).

However, up to now no in vitro test for eye irritation is available. Therefore, excluding this hazard may necessitate in vivo testing if other approaches (e.g., WoE) are insufficient or inconclusive (ECHA 2018a).

Strong prescriptions for record keeping and quality assurance procedures are a prerequisite for acceptance of data submissions to ECHA. Tests to identify intrinsic

adverse properties for human health in general must follow the principles of Good Laboratory Practices (GLP).

In an ideal world the test of first choice would be a definitive test. This type of test provides enough stand-alone information on dose response and adverse effects to make a final decision with regard to hazard assessment. However, in many cases, data from screening tests go first in a tiered testing strategy and definitive tests come only at a later stage. In special cases additional investigations are needed which do not follow a standard approach but are tailor-made for the hazard assessment of a specific substance. Those adjunct tests help to interpret the results of other tests and provide information useful for the hazard and risk assessment process (OECD 2005).

Physico-chemical data are often used for initial considerations of a specific hazard potential (see ▶ Chap. 8, "Characterization of Physicochemical Parameters in Toxicology"). Knowledge on selected properties (e.g., water solubility, acidity, alkalinity, hydrophilicity/lipophilicity, volatility) can foster an understanding whether a substance is bioavailable and to which extent it may be metabolized or be locally toxic.

Such data can also be used to support a read across strategy. For example, grouping of nanomaterials based on physico-chemical and toxicokinetic data should allow to fill data gaps for relevant hazard endpoints by reading across to another nanomaterial or the respective bulk material.

Quality Check of Gathered Information

All information must be assessed for its reliability, relevance, and adequacy.

Reliability is the inherent quality of the information as related to preferably standardized methodology and the way experimental procedure and results are described to give evidence of the clarity and plausibility of the findings. In general, the Klimisch code system allows ranking of the information for further review (see Table 2). The system consists of four categories (Klimisch et al. 1997):

1. Reliable without restrictions
2. Reliable with restrictions
3. Not reliable
4. Not assignable

In general, only Klimisch category 1 and 2 data are suitable for the hazard assessment of an endpoint. However, for the sake of completeness and transparency studies with a Klimisch code 3 or 4 should be included in the registration dossier.

"Relevance" is the extent to which data and tests are appropriate for a particular hazard assessment. Adequacy is the usefulness of the data for hazard and risk assessment purposes and combines reliability and relevance.

Highest relevance for hazard and risk assessment are reliable human data. However, in cases other than pharmaceuticals, human data generally are scarce.

Table 2 Klimisch codes/category of reliability

Reliability 1 **(reliable without restriction)** ● Guideline study (OECD, etc.) ● Comparable to guideline study ● Test procedure according to national standards (DIN, etc.)
Reliability 2 **(reliable with restrictions)** ● Acceptable, well-documented publication/study report which meets basic scientific principles ● Basic data given; comparable to guidelines/ standards ● Comparable to guideline study with acceptable restrictions
Reliability 3 **(not reliable)** more detailed free text ● Method not validated ● Documentation insufficient for assessment ● Does not meet important criteria of today standard methods ● Relevant methodological deficiencies ● Unsuitable test system
Reliability 4 **(not assignable)** short free text ● Only short abstract available ● Only secondary literature (review, tables, books, etc.)

A major pitfall for many human case reports is the lack of robust exposure data. Therefore, a dose-response analysis is rarely possible.

Experimental data, comparable in quality to those generated in controlled pharmaceutical clinical trials, are hardly available. Due to ethical concerns, testing on humans for hazard identification is not allowed. Controlled experimental studies may be used only as confirmatory tests for the assumption on safety made previously based on non-human data.

Poison control centers (PCC) may provide valuable data on the toxic potential of a product and its ingredients. At this point in time, discussion is ongoing how to make better use of the information collected at the PCC (Hahn and Feistkorn 2019).

Hazard Identification and Assessment

For the purpose of hazard assessment, the rules of the CLP regulation apply. These rules are described comprehensively and in detail in the chapter on classification and labeling of this booklet.

In this respect, the WoE approach is a guiding principle of REACH and CLP and is explained in detail in CLP regulation annex I:

> *"A weight of evidence determination means that all available information bearing on the determination of hazard is considered together, such as the results of suitable* in vitro *tests, relevant animal data, information from the application of the category approach (grouping, read-across), (Q)SAR results, human experience such as occupational data and data from accident databases, epidemiological and clinical studies and well documented case reports and observations. The quality and consistency of the data shall be given appropriate weight. Information on substances or mixtures related to the substance or mixture being classified shall be considered as appropriate, as well as site of action and mechanism or mode of action study results. Both positive and negative results shall be assembled together in a single weight of evidence determination."*

Table 3 Example for a weight-of-evidence approach

Step	Parameter	Finding	Action
1a:	Existing human or animal skin corrosion/irritation data	Skin corrosive	Classify as skin corrosive
1b:		Skin irritant	Classify as skin irritant
1c:		Not skin corrosive or skin irritant	Not classified
	No/ negative/ insufficient/inconclusive data		
2	Other, existing skin data in animals	Yes; other existing data showing that substance may cause skin corrosion or skin irritation	May be deemed to be skin corrosive or skin irritant
	No/negative/insufficient/inconclusive data		
3	Existing ex vivo/in vitro corrosivity data	Positive: Skin corrosive	Classify as skin corrosive
	No/negative/ insufficient/inconclusive data		
	Existing ex vivo/in vitro irritation data	Positive: Skin irritant	Classify as skin irritant
	No/insufficient/inconclusive data		
4	pH-based assessment (with consideration of acid/alkaline reserve of the chemical)	pH ≤ 2 or ≥ 11.5 with high acid/alkaline reserve or no data for acid / alkaline reserve	Classify as skin corrosive
	Not pH extreme, no pH data or extreme pH with data showing low/no acid/alkaline reserve		
5	Validated structure activity relationship (SAR) methods	Skin corrosive	Deemed to be skin corrosive
		Skin irritant	Deemed to be skin irritant
	No/insufficient/inconclusive data		
6	Consideration of the total **weight of evidence**	Skin corrosive	Deemed to be skin corrosive
		Skin irritant	Deemed to be skin irritant
7	Not classified		

Additional provisions are laid down in REACH annex XI, which provides options for meeting the information requirements in annex VII–X by other means than testing.

In a weight of evidence approach, different pieces of available information are weighted. This may be necessary when several reliable studies are available with conflicting results. Moreover, evidence from less relevant information is weighted for the hazard assessment. WoE is endpoint-specific and in general needs expert judgment.

Among the endpoints for which the WoE approach is frequently used is the hazard identification of local skin and eye effects which is demonstrated in the flow chart (Table 3). The flow chart follows the hierarchy of relevance with human

experience as the starting point and going down the road to in vivo data, in vitro data, pH, and alkaline/acidic buffer reserve, *and* QSAR predictions (ECHA 2017b).

The WoE approach grants flexibility and may reduce the costs and avoid animal tests. A major pitfall of the approach is that it is associated with lower legal certainty as compared to conducting a test.

Chemical Safety Assessment (CSA)

If a substance to be registered requires classification as dangerous or turns out to be persistent, bioaccumulative or toxic (PBT), or very persistent and very bio-accumulative (vPvB), the risk must be characterized in a chemical safety assessment. The exposure assessment and the risk characterization must be targeted at the specific hazard that had been identified, be it either for human health, physicochemical, environmental, or PBT or vPvB.

The chemical safety assessment is required for all substances subject to registration under REACH in quantities of 10 t or more per year per registrant. Its aim is to ensure that all risks are identified and under control (see Fig. 2) by relating exposure to threshold levels for hazards.

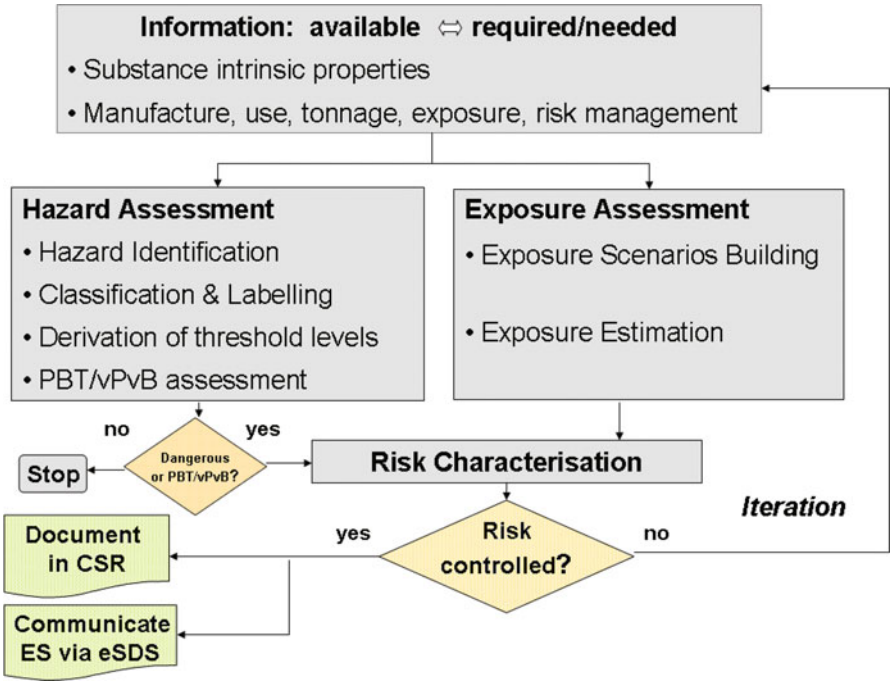

Fig. 2 Flow scheme for the chemical safety assessment

Exposure assessment is a tiered approach for which three levels are available. Entry into the process is possible at any of the three tiers. This *basic exposure assessment* can be used for all target groups (i.e., workers, general population [consumers, humans via the environment]). The tool commonly used for the basic assessment is the ECETOC targeted risk assessment (ECETOC 2004; ECETOC 2009). With relatively limited entry data results can be quickly obtained.

The next higher assessment tier can be for example *sector-specific generic exposure scenarios*. They are based on sets of exposure scenarios agreed along the supply chain between manufacturers and importers on one hand and downstream users (DUs) on the other hand. Risk management measures must be considered in order to ensure a use is safe to human health and the environment. Exposure scenarios are the communication tool to the user describing how to use a substance in a safe way. They are filed in the CSR together with other information and communicated to DUs via an annex to the safety data sheet (SDS), resulting in an extended safety data sheet (eSDS).

Whenever needed, *tier 3 specific assessments* may be conducted by modeling cases of specific applications as an outcome of joint efforts between supplier and downstream user. Exposure assessment may then be based on actual measurements from downstream use.

(For further background see the chapter on exposure assessment in this booklet – Chap. ▶ 30, "Exposure Scenarios in Toxicology").

The last phase of the chemical safety assessment is the risk characterization. Exposure levels must be compared to threshold doses or concentrations for which no adverse effects are expected. They are denoted as "derived-no-effect-levels (DNELs)." In cases where no thresholds can be derived, for example, for genotoxic carcinogens, a "DMEL" (derived minimum effect level) can be assigned. DMELs are defined as levels with a minimum risk that can be tolerated. The discussion below will focus on the DNEL concept, because this is the more commonly used.

DNEL setting must account for several aspects. All conditions of manufacturing or use must be addressed. Different factors must be applied depending on the target (workers, general population). Specific target organ toxicity data after acute and repeated exposure needs to be evaluated with special regard to the character of effects (systemic versus local).

The first step in the derivation of a DNEL is the decision on the initial point-of-departure (POD) value. Most frequently, the lowest reliable, relevant, and adequate no observed adverse effect level (NOAEL) from the registration data set is used as the POD. At this dose or concentration, no adverse treatment-related findings are observed. The NOAEL can be considered as the starting point for the risk assessment. The initial dose descriptor may require modification to address specific needs of the risk characterization. Assessment factors (AF, commonly referred by other regulatory jurisdictions as "modifying" or "uncertainty" factors) are then applied to the POD. Such factors address interspecies and intraspecies uncertainty and variability, exposure duration differences, dose-response considerations, and the overall quality of the dataset. The various AFs are multiplied, and the resulting product is

		ECETOC	ECHA TGD
Interspecies	rat–human	4	4
	‚remaining differences'		2.5
Intraspecies	Worker	3	5
	General population	5	10
Exposure Duration	28days → 90days	3	3
	90days → 2 years	2	2
	28days → 2 years	6	6
Route-to-route	Oral → inhalation		2

Fig. 3 Assessment factors for DNEL setting

used to divide the POD to derive a DNEL. The DNEL implies that humans should not be exposed to doses or concentrations above this level. Exposures below the DNEL are considered as "adequately controlled."

The ECHA technical guidance provides standard default assessment factors for DNEL setting. Several reviews showed that the ECHA factors are significantly more conservative than any other currently existing health benchmarks. Consequently, the DNELs derived, especially for the worker population are far lower than existing occupational exposure levels (OELs) utilized in Europe like the MAK and SCOEL values (Kreider and Spencer Williams 2010). Figure 3 provides a comparison of assessment factors as used, for example, by ECETOC and as described by the ECHA guidance (ECETOC 2010).

Risks are regarded as adequately controlled under REACH when the exposure levels to the substance are below the DNELs. This renders a "risk calculation ratio" (RCR). If risks are not under control, an iterative process starts. The chemical safety assessment must be refined, either by obtaining more data on the properties of the substance or by refining the exposure estimations to achieve more realistic estimates or implementing risk management measures.

(For further background see the following chapters: ▶ 6, "Toxicological Risk Assessment," ▶ 22, "Dose-Response Analysis: Identification of Threshold Levels for Chemicals," ▶ 23, "Extrapolation Procedures for Carcinogenic and Non-carcinogenic Compounds," ▶ 65, "Assessment of Limit Values in Regulatory Toxicology," ▶ 66, "Limit Value Setting in Different Regulatory Areas of Toxicology").

As a result of the risk characterization, the registrant defines the appropriate risk management measures to adequately control the risks. The measures are communicated down the supply chain by extended safety data sheets (eSDS). If for a specific use of a substance the risks are not under control, the supplier designates and communicates a use which he does not support. To this end Chapter 1.2 of the safety data sheet ("use advised against") is the right place for communication.

Exposure scenarios facilitate communication along the supply chain.

Table 4 Use descriptor system under REACH

Descriptor types		(Examples)
LCS	6 life cycle stages	IS: Use at industrial sites
SU	23 sectors of use	SU 11: Manufacture of rubber products
PROC	31 process categories	PROC 15: Use as a laboratory reagent
PC	43 product categories	PC17: Hydraulic fluids
AC	39 article categories	AC3–2: Electrical batteries and accumulators
ERC	25 environmental release categories	ERC6c: Production of plastics

Exposure scenarios are a set of conditions [usually based on a Process Category (PROC code) for workers, Product Category (PC Code) or Article Category (AC) for consumers or Environmental Release Category (ERC) for the environment] that describe how a substance can be safely used throughout its life cycle (see Table 4). Such scenarios include the necessary operational conditions (OCs) and risk management measures (RMMs). For each exposure scenario, the exposure levels of humans and the environment need to be determined. The exposure scenarios will cover all supported uses and life stages of the substance (ECHA 2011–2019).

If a downstream user notes that his own uses are not covered in the safety data sheet of his supplier, he can either contact his supplier in order to get the own uses covered or prepare his own CSA. To that end he may make use of the available information in the safety data sheet of the supplier.

The chemical safety assessment is documented in the Chemical Safety Report (CSR). A chemical safety report must always be current.

Evaluation

Registration dossiers are initially subjected to a technical completeness check which must be distinguished from the phase of dossier evaluation. A completeness check is conducted for each registration dossier before a registration number is allocated whereas the subsequent dossier evaluation ("compliance check") to assess the completeness and adequacy of the information provided is done on a spot-check basis by ECHA. Additionally, a substance evaluation is being performed for specific substances of potential high concern, being listed in the Community Rolling Action Plan (CoRAP). The criteria for substance selection combine both hazard and exposure aspects in a risk-based approach. Hazard related selection criteria comprise for example endocrine disruption, CMR, and sensitizing properties (see Table 5).

The responsibility for this evaluation is with the different member states which scrutinize whether a substance constitutes a risk to human health or the environment. In contrast to the compliance check, information requested from registrants during a substance evaluation typically goes beyond the standard requirements of REACH as they are needed to clarify a specific concern. Based on the outcome of the substance

Table 5 Evaluation processes under REACH

Item	By whom?
Technical completeness check	ECHA (automatically with manual verifications by ECHA staff)
Dossier evaluation (ECHA) – Compliance check	ECHA
Substance evaluation (CoRAP)	Competent authorities of the member states
Evaluation of test proposals (annex IX und X)	ECHA

evaluation, a follow-up regulatory action such as a need for harmonized classification and labeling or the need to start authorization or restriction processes might be identified by the respective competent authority.

Authorization and Restriction

Authorization and Restriction are the risk management instruments of the competent authorities of the member states and the EU commission under REACH.

The REACH regulation intends to filter out substances of very high concern (SVHC) by controlling their use. Temporary authorizations are granted under certain conditions of uses while working towards their substitution. High concern is raised by substances that are CMRs, PBTs, or vPvBs. Apart from these SVHCs which are explicitly mentioned in the regulation, article 57 contains an opening clause that enables competent authorities to carry out individual case-by-case examinations for further substances. In cases of "scientific evidence of probable serious effects to human health or the environment which give rise to an equivalent level of concern," these substances may also be proposed for authorization. Candidate substances for this procedure are endocrine disruptors.

Authorizations of uses for the placing on the market are granted by the Commission if the risks arising from their use are adequately controlled, in principle when derivation of limit values is possible. In those cases where a substance causes an unacceptable risk to human health or to the environment, its substitution must be considered. The considerations account for suitable alternatives, if available, that are economically and technically feasible. In cases where no adequate control can be ensured (e.g., non-threshold substances), a socio-economic analysis (SEA) must be prepared, demonstrating that the benefit of further use of the substance outweighs societal risk.

Authorized substances are generally banned except for some specific uses, which have been authorized by the EU Commission after application. Companies must apply for exemptions and provide a justification for a continuous use.

In contrast, the *restriction* of substances does not represent a total ban but the prohibition of certain uses. Restriction can be understood as a measure from authorities to manage situations where specific risks are not adequately controlled.

Learnings

Up till now approximately 98,500 dossiers have been submitted for about 22,800 substances by 15,300 companies. This equals total registration costs of up to 3 billion Euro (Haider 2019).

The processes of substance evaluation and risk management are ongoing. As of December 2019, 376 substances have been included in the CoRAP list. The evaluation of 117 substances has been completed. 201 substances have been included in the SVHC list and the authorization list already comprises 43 substances.

So far, 73 restrictions for 70 different substances have been adopted under REACH.

After 10 years of REACH, the Commission did a review of the achievements and concluded that "the REACH regulation functions well, delivers results and addresses citizens" concerns about chemical safety. However, the report states that further improvements are needed to make the legislation more efficient – especially for the evaluation, restriction and authorization processes. The report sets out actions for ECHA, the Member States, Commission, and industry (ECHA 2018b).

A recent study on REACH compliance by the German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung – BfR 2018) emphasized the need for improvement. The BfR study indicated that at least one third of the registrations reviewed in the project are considered as non-compliant because they use insufficient alternative data or data waiving to cover higher tier endpoints. It is not surprising that the outcome of the BfR conclusion triggered controversial discussions between industry and authorities. As the regulation allows animal testing only as the last resort, many registrants used derogations/data waivers to avoid testing. Uncertainty seems evident in two aspects: *safety* uncertainty because important data may be missing that impacts the risk characterization and *legal* uncertainty given the "complaint of non-compliance" by the authority to registrants that used derogations/data waivers for animal welfare and cost saving reasons.

A major issue when REACH was introduced was the need for animal testing to obtain data on the hazard potential of chemicals. The ECB estimated the number of animals required for the phase-in substances at 40 million animals (European Commission 2006).

This scary number led to several provisions in the regulation to reduce animal use, mainly by opening options for alternatives in testing and evaluation. Indeed, ECHA rapidly took responsibility to embrace newly developed alternative methods as soon as they were available and accordingly amended the requirements of the annexes VII through X. Both in vitro tests for local irritation and corrosion on skin and eye effects were included in the standard data set. They (partly) replaced the in vivo methods. The same applies to skin sensitization, for which now a set of five in vitro tests is available and must be used as the starting point by the registrant. These approaches save the life of 6 rabbits (irritation) or 16 mice (sensitization). However, tests with high animal use (repeated dose testing, reproductive and developmental toxicity, carcinogenicity) currently lack accepted test alternatives. One exception to be mentioned here is the replacement of the two-generation reproductive toxicity

study by the EOGRT ("Extended one generation reproductive toxicity study"), which significantly reduces the number or animals used per test: 1400 instead of 2600.

Before the adoption of REACH alternative *assessment* strategies were regarded as a salvage pathway (European Union 2006b). Especially, the weight of evidence approach including read-across, QSAR modeling, and exposure-based waiving (TTC-concept) were ideas to reduce the number of tests with high animal consumption. However, experience from the first 10 years shows that waivers are increasingly challenged by the evaluating authorities.

(Further background can be found in the following chapters: ▶ 14, "Computer-Based Prediction Models in Regulatory Toxicology," ▶ 12, "Toxicity Testing In Vitro: Regulatory Aspects").

Statistics on the number of animals used so far due to the REACH requirements are not available.

With the trend not to accept waivers, it is questionable whether the target to reduce animal use as defined in the 2006 JRC statement will be reached.

Outlook

The European Commission intends to amend the regulation to increase dossier compliance. The future target for ECHA will be to evaluate 20% (instead of 5%) of all registration dossiers in each tonnage band which equals to about 30% of all registered substances. Whilst ECHA is focusing on dossier evaluation, registrants are requested to steadily update their registration dossiers with new information.

One of the major focus areas of substance evaluations scheduled for the next years are endocrine disruptors (EDs) which are eligible as being identified as SVHC based on equivalent level of concern as for PBT and CMR substances.

While the evaluation of ED properties in the REACH dossiers is currently based on available study data, for example, higher tier reproductive toxicity studies, the European Commission proposed to update the regulation to include specific testing requirements for ED including the need for a formal ED assessment in the dossier. However, no conclusion on these proposals has been reached yet.

Other topics currently discussed for potential including in the regulation are registration requirements for polymers, which are currently exempted from the regulation.

There is no doubt that REACH is having global implications. Many regulatory jurisdictions outside of Europe have been adopting new regulations that model after REACH. As part of the process of harmonization with European law, in 2017 Turkey published a chemical regulation that is 95% identical with the European REACH regulation. It requires all substances imported or manufactured in/into Turkey to be registered by 2023. In contrast to EU REACH, there are very strict requirements around the chemical safety assessment (CSR). Any chemical safety assessments must be done by a certified expert, trained by an accredited institution. The requirements of certification are clearly defined in the regulation. Like Turkey, Korea

published a REACH-like regulation with a final registration deadline for substances above 1 t in 2030. While the main requirements are similar to the EU REACH, some specifics are different. For example, Korea requires the registration of polymers, which are currently exempted from the EU REACH.

REACH is an unprecedented chemical legislation, which continuously increases the safety information on chemical substances. Communication along the supply chain is intense. Numerous new threshold values have been and continue to be generated. More substances than in the past are under discussion at the same time due to newly identified hazard properties. Evaluation leads to broad-scale legislative actions, be it new hazard classifications with corresponding impact to downstream legislation or REACH-related, authorizations, and restrictions.

REACH is a highly complex regulation which requires and will continue to require huge endeavors from all impacted stakeholders.

Cross-References

- ▶ Assessment of Limit Values in Regulatory Toxicology
- ▶ Characterization of Physicochemical Parameters in Toxicology
- ▶ Computer-Based Prediction Models in Regulatory Toxicology
- ▶ Dose-Response Analysis: Identification of Threshold Levels for Chemicals
- ▶ Exposure Scenarios in Toxicology
- ▶ Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds
- ▶ Importance of Physicochemical and Physical Properties for Toxicological Risk Assessment
- ▶ International Regulation of Toxicological Test Procedures
- ▶ Limit Value Setting in Different Regulatory Areas of Toxicology
- ▶ Toxicity Testing In Vitro: Regulatory Aspects
- ▶ Toxicological Risk Assessment

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Assessment of Mixtures: Bridging Principles and Other Approaches

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Walter Aulmann and Nathan Pechacek

Contents

Abstract

In general, test data for mixtures are available only in rare cases. For a hazard assessment, a common surrogate method is to delineate their hazard potential from data of individual ingredients. Another approach exists to extrapolate from mixtures with test data to nontested mixtures. Such bridging principles have been in use in certain sector-specific applications already in the past. With the adoption of the new globally harmonized system for classification and labeling of chemicals (GHS), bridging principles gained a broad regulatory acceptance for the hazard assessment of mixtures. In order to make use of the principles, strong formal requirements must be fulfilled. This chapter describes the various applicable principles, such as the principle of "dilution," "concentration," "interpolation," and "batching" of "substantially similar mixtures."

W. Aulmann (✉)
Ecolab Deutschland GmbH, Monheim, Germany
e-mail: walter.aulmann@ecolab.com

N. Pechacek
Ecolab Inc, Eagan, MN, USA
e-mail: nathan.pechacek@ecolab.com

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Another way for the assessment of a mixture is the weight of evidence approach leading to an expert judgment. It is less formal, but it suffers from legal certainty and may be challenged more frequently by third parties.

Keywords

GHS – global harmonized system · Additivity principle · Bridging principle · Weight of evidence · Dilution · Concentration · Batching · Interpolation · Substantially similar mixture · Minor modification of mixtures

Abbreviations

BfR	Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung)
C&L	Classification and labeling
CMR	Carcinogenic, mutagenic, reproductive toxicant
CSR	Chemical safety report
DNEL	Derived No-Effect Level
DU	Downstream user
ECHA	European Chemicals Agency
ES	Exposure Scenario
eSDS	Extended Safety Data Sheet
GHS	Globally harmonized System of Classification and Labelling of Chemicals
GLP	Good Laboratory Practice
IUCLID	International Uniform Chemical Information Database
NOAEL	No Observed Adverse Effect Level
PBT	Persistent, Bioaccumulative, Toxic
PCC	Poison control centers
POD	Point-of-departure
RMM	Risk Management Measures
SEA	Socio-economic analysis
SIEF	Substance Information Exchange Forum
SME	Small or Medium Enterprise
SVHC	Substances of Very High Concern
vPvB	Very Persistent very Bioaccumulative

Introduction

Test data on mixtures are scarce. This is due to the fact that the number of mixtures on the market by far exceeds the number of single substances: the world of chemistry is mainly a world of mixtures. To make best use of limited resources and to limit animal tests to a minimum, mixtures are basically assessed on their toxicological hazard by other means than direct testing.

EU Regulation (EC) No 1272/2008 on the classification, labeling, and packaging of substances and mixtures ("CLP Regulation") incorporates the concept of using reference mixtures by suggesting the following options:

- Additivity principle
- Evaluation of the degree of modification of a nontested mixture as compared to the reference mixture (concept of "minor modification")
- Assessment of mixtures based on existing toxicological data on similar mixtures (i.e., bridging principles)
- Weight of evidence and expert judgment

The Additivity Principle

A commonly used approach is modular: Toxicity is evaluated based on the toxicological properties of the individual components and their mass content in the mixture. This approach follows general conventions which are laid down under the globally harmonized system. In the EU, they were adopted under the Regulation on Classification, Labelling and Packaging (CLP). They are known as "additivity principles." For more details, consult the CLP regulation and also additional guidance in this booklet, see ▶ Chap. 68, "Health Hazard Classification and Labelling".

In those rare cases where test data on mixtures are available, rules exist to extrapolate from a mixture for which the hazard must be assessed to a tested mixture.

Extrapolation needs a comprehensive technical justification. At a practical level, a reference mixture is defined as a mixture of known composition which has been tested for a toxicological or ecotoxicological endpoint of interest (e.g., skin or eye irritation/corrosion). Both the reference mixture and the untested mixture must be well characterized to allow a classification decision. Chemical characterization includes data on relevant physicochemical properties, such as the pH of the mixture and its buffer reserve (alkalinity/acidity) in the case of pH extreme formulations (see also ▶ Chap. 8, "Characterization of Physicochemical Parameters in Toxicology"). Well-documented data on the toxicological properties on the mixtures and their ingredients must be comprehensive and reliability-checked (see also ▶ Chap. 76, "REACH and CLP: Its Role in Regulatory Toxicology").

The Concept of "Minor Modifications"

A separate and complete toxicity assessment of a modified mixture does not need to be carried out if the modification is only minor in comparison to a classified reference mixture for which toxicity data are available. In this context, "minor" means that the modification lies within the permitted variations for the initial concentrations of the hazardous constituents, as shown in Table 1.

Table 1 Permitted variations for consideration as a minor modification

Initial concentration Range of the constituent	Permitted variation in initial concentration of the constituent
<2.5%	±30%
2.5% < C < 10%	±20%
10% < C < 25%	±10%
25% < C < 100%	±5%

If the variations in the modified mixture exceed the limits, they no longer can be considered "minor." Then, the mixture should be evaluated with concepts other than the "minor-modification principle."

Bridging Principles

In the absence of adequate toxicity information for a mixture *as such,* it should be considered whether available toxicity data from *similar* mixtures can be used. The underlying concept encompasses the comparison (i.e., "bridging") of the available technical information. The comparison endpoint is specific. In other words, a bridging may, e.g., be possible for local skin effects, but not necessarily also for systemic repeated dose toxicity.

The following "bridging principles" are part of the Globally Harmonized System (GHS – United Nations 2005)/European Classification, Labeling and Packaging Regulation (CLP – European Union 2008):

- *Concentration* of highly hazardous mixtures – Where a mixture is already classified in the highest hazard category, then it can be assumed that a more concentrated mixture will also be in the highest hazard category.
- *Dilution* –The test mixture is diluted with a substance (diluent) that has the same or a lower hazard category than the least hazardous ingredient in the original mixture. In this case it can be assumed that the respective hazard of the new mixture is equivalent to that of the original tested mixture.
- *Interpolation* within one toxicity category – defines how much the concentration of a hazardous mixture may vary without changing the classification. Usually two reference mixtures are needed which are broadly similar and in the same hazard category for a given endpoint. Typically, a third mixture has the same hazardous constituents in concentrations that are between those of the two reference mixtures and can be classified the same as the reference mixtures. It is noted that this approach may allow greater variation for individual constituents than those permitted in the "minor" "modification method" highlighted in Table 1.
- *Substantially similar mixtures* – this applies if constituents with the same hazard category and the same potency are exchanged in a mixture. In this case, the hazard category of the mixture does not change. Potency may be expressed by specific concentration limits: If two ingredients are in the same hazard category, e.g.,

category 2 – "severe eye irritation," but the specific concentration limits are, e.g., 5% and 20%, then they do *not* have the same potency. In this case, the principle of "substantially similar mixtures" cannot be applied.

- *Batching* – If a batch of a mixture is produced under a controlled process, then it is assumed that the hazards of each new batch are equivalent to those of previous batches. This method cannot be used where there is significant variation between batches which may affect hazard classification.

Long before the adoption of GHS and CLP, such bridging principles had been tried and tested in certain chemical sectors like the detergents industry (AISE 1993). They gained broad, but not unanimous acceptance in the regulatory community. With CLP, a strong basis and higher legal certainty as compared to the past were achieved across the overall European Union and beyond.

Experience over decades showed that "Interpolation within one toxicity category" and "substantially similar mixtures" are the bridging principles mostly used in the day-to-day classification business.

Further details on the use of bridging principles are given in the CLP Regulation and in the Guidance on the application of Regulation (EC) No 1272/2008 (ECHA 2017).

Weight of Evidence (WOE) Approach and Expert Judgment

Generally, the criteria for using the bridging principles for the classification of mixtures are strict. Often, they are not applicable for formal reasons. Therefore, the use of expert judgment to decide on the classification or nonclassification of a mixture can be helpful.

Expert judgment requires knowledge of the chemistry and toxicological profile of the product categories in question. Also, expertise is required to weigh the relevance of different types of test systems. The latter is particularly important when the classification of the reference mixture is based on heterogeneous data sets including data from scientifically valid but not fully validated methodologies or in cases where conflicting information is available.

Classification of a mixture based on WOE requires the consideration of all available information bearing on the determination of a given health endpoint. This includes results of suitable in vitro tests, relevant animal data, chemical category information, quantitative structure activity relationship (QSAR) results, and human experience taken from occupational, epidemiological, clinical studies, and well-documented case reports. The quality and consistency of the information must be given appropriate consideration. Positive and negative results should be collected in a single WOE determination (see ▶ Chap. 76, "REACH and CLP: Its Role in Regulatory Toxicology").

The priority given to different pieces of information is generally determined on a case-by-case basis. Some guidance is provided by the CLP Regulation to address conflicting findings (European Union 2008). From this guidance, basic qualitative rules can be established for the classification and labeling of mixtures:

1. When several studies with conflicting results are available for one reference mixture, the quality and reliability of the studies, as well as their relevance for classification and labeling, must be taken into account. Toxicity data derived from in vitro and in vivo studies are generally viewed as acceptable if they receive a reliability score of 1 or 2 according to the Klimisch criteria (Klimisch et al. 1997). When the reliability of studies is comparable, studies considered the most relevant for the particular hazard are given more weight in the assessment.
   In general, the following relevance hierarchy applies:
   human studies > animal studies > in vitro studies > QSAR studies
   However, deviation from this hierarchy may occur on a case-by-case basis when study specifics are considered. Hence, the final decision, which studies are most relevant, is subject to expert judgment.
2. In case, the study results of two or more reference mixtures are in conflict, the degree of similarity of the reference mixture to the mixture to be assessed, in addition to the factors mentioned above, will need consideration. The data from the reference mixture that is judged to be the closest to the comparison mixture under assessment should be given the greatest weight.
3. In cases of no appreciable differences in study reliability or ranking, the study giving rise to the highest concern should be taken as the key study for the classification of the comparison mixture.

Outlook

Over the last two decades in vitro methods were adopted especially for eye and skin. They provide new tools to overcome the initial imperative not to test mixtures: Animal welfare considerations do not play a role when applying in vitro methods.

Not surprisingly, the new in vitro methods are increasingly used also for the testing of mixtures. Today, e.g., for the investigation of skin effects a full battery is available to enable a decision whether a mixture is either corrosive, irritant, or does not require hazard classification. However, caution is needed because experience with the new methods is limited. The applicability of the new methods for the plethora of substances still needs to be proven, given the fact that the validation trials included only a limited number of substances and mixtures.

Toxicologists must keep in mind the frequently limited data base which leads to the adoption of in vitro tests. No doubt, they offer new opportunities. However, toxicologists need to continue to collect and analyze the test results and compare them with gold-standard data (human or in vivo) to increase confidence that the methods are applied appropriately in every case.

Cross-References

► Characterization of Physicochemical Parameters in Toxicology
► Health Hazard Classification and Labelling
► REACH and CLP: Its Role in Regulatory Toxicology

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Notification of Hazardous Mixtures and Cosmetic Products for Poisons Centers in the European Union

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Herbert Desel, Esther Feistkorn, Ronald de Groot, and Pieter Brekelmans

Contents

Abstract

Poisons centers (PC) support medical management of poisoning cases in all parts of the health system. Most often, PC are involved in emergency cases, providing a rapid clinical risk assessment (RCRA). RCRA includes identification of products the patient is exposed to, retrieval of their chemical formulas, compilation of specific information on the toxicity of the ingredients, and evaluation of the patient's individual exposure and condition. The result of the RCRA forms the basis for advices on medical management of the poisoning case. To perform

H. Desel (✉)
Department Exposure, Unit 32, Exposure Assessment of Hazardous Products, German Federal Institute for Risk Assessment, Berlin, Germany
e-mail: Herbert.Desel@bfr.bund.de

E. Feistkorn
Exposure Assessment of Hazardous Products, German Federal Institute for Risk Assessment, Berlin, Germany
e-mail: Esther.Feistkorn@bfr.bund.de

R. de Groot · P. Brekelmans
National Poisons Information Center (NVIC), University Medical Center Utrecht, GA, Utrecht The Netherlands
e-mail: R.degroot-5@umcutrecht.nl; P.Brekelmans@umcutrecht.nl

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RCRA adequately, it is crucial that PC receive information on the composition and toxicological properties of products placed on the market. To ensure this, submission of information on hazardous mixtures and cosmetic products by companies to PC is a legal obligation in all EU Member States. Harmonized notification formats and procedures and two modern European database systems, i.e., the European Chemicals Agency (ECHA) Poison Centre Notification (PCN) Submission Portal (PCNP) and Cosmetic Products Notification Portal (CPNP), help to increase dataset quality and to reduce notification workload for industry.

Keywords

Notification of hazardous mixtures · European harmonized notification · Poison centers · Poison Centre Notification · ECHA Submission Portal · PCN format · Cosmetic Products Notification Portal · Risk assessment · Clinical toxicology · Regulatory toxicology · Toxicovigilance

Introduction

Poisons centers provide information on poisons and give medical advice in cases of accidents with substances, including toxins and synthetic chemicals. For this purpose, they need all the available information on the often complex ingredients of chemical products and cosmetic products.

The European Commission has harmonized information requirements for mixtures classified as hazardous for human health or physical effects becoming mandatory in 2021 or 2024. Importers and downstream users shall submit this mixture information to Member States, to be used by their poison centers for emergency health response.

EU Cosmetics legislation requires that cosmetic products to be marketed in the EU must be registered in the Cosmetic Products Notification Portal before being placed on the market since 2013. This portal is making this information available to poison centers for the purposes of medical treatment and for enforcement authorities for market surveillance.

Poisons Centers Have an Important Role in Clinical and Regulatory Toxicology

Today, about 80 poisons centers (poison control centers, poison information centers, PC) play an important role in toxicological risk assessment and management of human (and animal) poisonings in Europe, especially in emergency medicine. Worldwide, about 200 PC are registered in a directory of the World Health Organization. PC either are independent public institutions or are affiliated with a (university) hospital or an authority.

In PC, toxicologically trained medical doctors and other experts (specialists in poison information (SPIs), e.g., pharmacists or nurses) provide information to general practitioners, medical doctors at the hospital, or other professional healthcare

personnel on poisoning risks, clinical symptoms, and medical treatment of patients exposed to a toxic agent. As an intoxication progresses and/or if a patient is admitted to a hospital, often several calls are needed to complete the toxicological risk assessment, medical diagnosis, advice on the individual poisoning management plan, and case recording.

In most countries, PC also give advice directly to the exposed patient or to family members, especially if children are exposed. An "all-clear" can be given in the majority of these cases and thus saves thousands of unnecessary presentations to the emergency medical system every day (European Commission Services 2020).

PC are often contacted by local, national, and European authorities and by industry to report on their experiences with specific poisonings. To answer these questions – and to analyze cases for quality assessment of the service and for medical studies – PC register all exposure cases in local or national case databases. PC case databases are recurrently analyzed to describe poisoning frequencies, detect new poisoning risks, and identify poisoning trends, e.g., novel poisoning risks arising from new agents and products in an early stage (*toxicovigilance*).

Notification of Product Information for Poisons Centers Facilitates Rapid Clinical Risk Assessment

The most important part of the PC service is to perform or to support clinical risk assessment. Together with findings of the physical examination of the patient and in ambiguous cases the results of toxicological lab investigations, the risk assessment constitutes the basis for correct medical diagnosis and subsequent medical decisions on patient treatment and monitoring.

To facilitate toxicological risk assessment after exposure to a potentially hazardous commercial product, access to toxicity-related product information, especially detailed information on the product formula, is needed. Two European Union (EU) regulations are directed to harmonization and centralization of product notification in EU Member States.

Harmonized Notification of Hazardous Products According to CLP Regulation (EC) No. 1272/2008, Article 45 and Annex VIII

Article 45 of the Regulation on classification, labelling, and packaging of substances and mixtures (EU) No. 1272/2008 (CLP Regulation) states that poisons centers shall have at their disposal all product information needed to carry out "the tasks for which they are responsible," i.e., mainly for clinical risk assessment in emergency cases (European Parliament and Council 2008 Regulation (EC) No 1272/2008).

For this purpose, all EU Member States had to appoint a body or bodies responsible for receiving this information including "the chemical composition of mixtures placed on the market and classified as hazardous on the basis of their health or physical effects." The appointed body, i.e., a governmental authority in most Member States or a PC, continuously collects product information notified by duty-

holding companies, i.e., "importers and downstream users." Product data have to be kept confidential and must not be used for any purpose except (1) medical management of poisoning cases or (2) statistical data analysis to facilitate poisoning prevention if requested by the Member State (de Groot et al. 2017).

However, Article 45 does not describe in detail what information is required and how it should be notified. This shortcoming was recognized at a late stage in the development of the CLP Regulation, leading to introduction of Paragraph 4 to Article 45: following this paragraph, the European Commission Services carried out a review to see whether European harmonization of product notification is feasible. The review process included consultations with relevant stakeholders such as the European Association of Poisons Centres and Clinical Toxicologists (EAPCCT), industry associations, and national authorities from 2009 to 2011. After a positive outcome, the European Commission Services, together with all stakeholders, developed Annex VIII to the CLP Regulation, released as Regulation (EU) No 2017/543. Annex VIII defines the procedures and information requirements for harmonized electronic product notification in all EU Member States. These requirements applies in 2021 for mixtures for consumer and professional use and for mixtures with industrial use in 2024. Advised by the Regulation, the European Chemicals Agency (ECHA) has developed the technical Poison Centres Notification (PCN) format for submission of the required information.

The main features of the Annex VIII/PCN format are:

1. In contrast to the requirements for Safety Data Sheets, also non-classified ingredients shall be notified if present above a threshold concentration.
2. Concentration of ingredients in a mixture can be notified using flexible ranges with well-defined width.
3. A Unique Formula Identifier (UFI), a 16-digit alphanumeric code, that has to be printed on or affixed to the product label, is assigned to each mixture composition dataset.
4. The format contains an intended use product category for each product; a European Product Category System (EU-PCS) was developed for this purpose.
5. The PCN format is described by Extensible Markup Language (XML) scheme.

The PCN format is a subset of IUCLID, a format developed and maintained by OECD in close collaboration with the ECHA, used for the registration of substances according to the REACH Regulation. For further facilitation of product data submission, the "ECHA PCN Submission Portal" (PCNP) was established in 2019. It allows industry to upload notification dossiers or to prepare and submit notification dossiers online. In addition, the portal performs a technical validation of the submissions and distributes the datasets to those Member States' appointed bodies addressed by the submitter. The ECHA Submission Portal is accessible via ECHA's "Poison Centres Website" (European Chemicals Agency (ECHA) 2020).

As late development and based on a European Commission's decision, ECHA has developed and is maintaining a searchable database containing all Poison Centre

Notifications submitted for each Member State, the so-called "Poison Centre Notification (PCN) Database."

The Cosmetic Products Notification Portal

According to Article 13 of the Cosmetics Regulation (EC) No. 1223/2009, there is an obligation for all manufacturers and importers of cosmetic products to notify product information to a central European database, the "Cosmetic Products Notification Portal (CPNP)" (European Parliament and Council 2009 Regulation (EC) No 1223/2009). The CPNP is located at the European Commission Services in Brussels. Poisons Centres of all EU Member States have access to the complete CPNP dataset; competent national authorities have access to a data subset needed for market surveillance. PC can either access the CPNP online or download datasets for import into local PC product databases. Local product data download enables reliable and easy linkage between poisoning case documentation and product formula in the PC databases to facilitate reporting (toxicovigilance, see above) (European Commission Services 2002a (CPNP) – Cosmetics – Consumer Affairs).

Notification to the CPNP is performed online through a secured website. One out of three different formats can be chosen:

1. Frame formulations (FF) for products containing only ingredients of low toxicological concern under most exposure conditions. FF are indicating maximum concentrations for ingredients. The set of frame formulations was defined and is maintained by a working group of the European Commission, Poisons Centres, and cosmetic industry.
2. FF plus indication of exact concentrations for ingredients with moderate or high toxicity.
3. Notification of full formula with exact concentration for all ingredients.

Besides the formula, further data have to be submitted, e.g., exact and complete product name(s) in all relevant languages, pH value, and image of the packaging. There are strict rules for when an update of product information is required (European Commission Services 2002b (CPNP) – User Manual).

Cross-References

- ▶ Dealing with Diseases That Have Been Attributed to Chemical Exposures
- ▶ Exposure Scenarios in Toxicology
- ▶ Health Hazard Classification and Labelling
- ▶ National and International Collaboration in Regulatory Toxicology
- ▶ REACH and CLP: Its Role in Regulatory Toxicology
- ▶ Sensitive Humans Versus Average Persons in Toxicology
- ▶ Teaching Chemical Safety

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Recommended Reading

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Part VII

Regulated Areas

Chemical and Biological Weapons and Their Regulation

79

Horst Thiermann, Kai Kehe, Julia Riehm, and Lothar Zöller

Contents

Abstract

Chemical and biological agents have been used as weapons since ancient times. But it was only after the disastrous use of this type of agents in World War I that international efforts were made to prohibit them. These efforts were very

H. Thiermann (✉)
Bundeswehr Institute of Pharmacology and Toxicology, Munich, Germany
e-mail: HorstThiermann@bundeswehr.org

K. Kehe
Department F – CBRN Medical Defense, Military Medical Research and Development, Bundeswehr Medical Academy, Munich, Germany
e-mail: KaiKehe@bundeswehr.org; kai.kehe@lrz.uni-muenchen.de

J. Riehm · L. Zöller
Bundeswehr Institute of Microbiology, Munich, Germany
e-mail: JuliaRiehm@bundeswehr.org

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successful and continue until today. But nevertheless, it was not possible to forestall their use completely as shown recently by the events in Syria and most probably the attack on Kim Jong Nam. This chapter gives a short introduction in the field. It also characterizes some important agents and outlines, what is necessary to be prepared against a possible attack.

Introduction and History

Chemical warfare agents are chemicals, which have a very high toxicity and may therefore be misused as weapons to cause death or disease among the target population. For historical reasons, the term "chemical warfare" agent includes synthetic chemicals (toxicants) but usually does not include the toxins, which are poisons produced by living organisms. Toxin agents are often taken as a subgroup of biological agents (see below). However, for the toxicological risk assessment, there is no basic difference between toxicants and toxins. The disabling effect of such weapons on target persons is horrific. It is in the nature of such agents that they will without differentiation affect the exposed population.

It is probably a result of the widespread use of chemical weapons during World War I that international efforts were made, to restrict and ban such agents. In 1925, the use of asphyxiating, poisonous, or other gases and of bacteriological methods of warfare was prohibited and included in the Geneva Protocol. Mandatory regulations regarding the possession and development of warfare agents followed in 1968 (Chemical Weapons Convention) and 1972 (Biological Weapons Convention). On 29 April 1997 the Chemical Warfare convention entered into force, and 193 (data from today, one more nation is a member). Despite these regulations, several offenses occurred. The exile Bulgarian Markov died after an attack with ricin toxin in London in 1978. About 10 years later, members of the Japanese Aum Shinrikyo cult tried to poison attendants of a royal wedding party spraying medium supernatant from cultures of neurotoxin-producing *Clostridium botulinum* strains. According to the American "Working Group on Civilian Biodefense," 19,000 l of botulinum neurotoxin were produced during the 1990s in Iraq. Officially, there are no existent biological warfare programs nowadays. However, their presence cannot be completely denied as there are no legal control mechanisms. In 1995, the sarin subway attack was of terrorist origin. Such an attack is able to scare a whole nation and has high impact on politics and decision-making. In 2013, ricin toxin was used in a bioterror attack in the United States when three series of letters containing the substance were sent to officials and even the President. Although nobody was injured, the news attracted public attention and intensive media coverage worldwide. Chemical warfare agents are likely to be used in terrorist attacks as they are relatively easy to produce and designed to have a high lethality.

The use of poison in military conflicts is very old. One of the first attempts to use toxic substances in military operations was during the Cirraean war [595–585 BC].

The city of Kirrah was attacked by the Amphictyonic League of Delphi. A secret water supply of the city was poisoned with *Helleborus* roots. Helleborin caused severe diarrhea and weakened the defenders of the city. This is believed to be the first report of chemical warfare. Later in history, more toxic substances have been stockpiled and used as chemical weapons.

For example, historical documents claim the Assyrians to consciously poison their enemies by the application of *Claviceps purpurea*'s ergot in the sixth century BC. Later in time, one of Hannibal's warfare strategies aimed at throwing poisonous snakes on Pergamenes' ships.

Chemical warfare agents are still stockpiled and available for military use. After the last chemical war between Iran and Iraq 30 years ago, there was a long lag-period, in which there was no proof for the use of chemical or biological weapons in war. However, the situation changed dramatically, when in August 2013, news on a possible use of chemical weapons in a populated area in Syria made the headlines. Meanwhile, the use of sarin and sulfur mustard during the Syria crises was reported by the OPCW as having been verified and responsible for more than 1000 victims.

Chemical Weapons

Definitions

Article II of the Chemical Weapons Convention (CWC) defines a *toxic chemical* as "any chemical which through its chemical action on life processes can cause death, temporary incapacitation or permanent harm to humans or animals." *Toxic chemicals* and/or devices (munitions) to disperse toxic chemicals are regarded as *chemical weapons*. Toxic chemicals, synthesized for military purposes, used in this context, are also called chemical weapon agents (CWAs). *Old chemical weapons* are produced before 1925.

CWAs are commonly classified as blood, blister, nerve, psychological, and pulmonary agents. This classification is commonly used but scientifically not correct, e.g., blood agents do not solely react with blood constituents. Blister agents may cause (more severe) systemic poisoning.

The CWC Annex of Chemicals distinguishes so-called Schedule 1–3 chemicals, which are regarded as CWAs.

Schedule 1 substances are toxic chemicals which have been used as chemical weapons or may be used for manufacturing chemical weapons (Table 1). Their civil use is limited. Some of the Schedule 1 chemicals have limited use in medicine or research. Saxitoxin and ricin are also Schedule 1 substances.

Toxic chemicals with possible use as chemical weapons or in their manufacturing process and which have legal use as well are listed in Schedule 2 (small-scale applications) and Schedule 3 (large-scale applications).

Table 1 Examples of chemical warfare agents listed in Schedule 1 and their physicochemical properties

Substance [NATO code]	Chemical name	CAS	MW	Boiling point [°C]	Freezing point [°C]	Vapor pressure [mmHg at 20 °C]	Vapor density [air = 1.0]	Solubility in water [g/100 g H_2O, 20 °C]
Schedule 1								
Sarin [GB]	Isopropyl methylphosphonofluoridate	107-44-8	140.1	158	−56	2.1	4.9	Miscible
Soman [GD]	Pinacolyl methylphosphonofluoridate	96-64-0	182.2	167–200	−42	0.4	6.3	2.1
Tabun [GA]	Dimethyl amidocyanoethylphosphate	77-81-6	162.1	220–246	−50	0.037	5.6	9.8
Cyclosarin [GF]	*O*-Cyclohexyl-methylfluorophosphonate	329-99-7	180.2	239	−30	0.044	6.2	0.37
VX	S-(2-diisopropyl aminoethyl) *O*-ethyl methyl phosphonothiolate	50782-69-9	267.4	298	−51	0.0007	9.2	3
HD	Bis(2-chloroethyl) sulfide	505-60-2	159.1	227.8	−50	0.072	5.6	<1

Characteristics of Chemical Weapon Agents (CWAs)

Nerve Agents

Organophosphorus (OP) compounds are widely used pesticides in agriculture. More than 160,000 deaths after OP poisoning occur worldwide. The main causes are of suicidal nature or accidents. A subgroup of OP compounds has highly toxic properties and was stockpiled as chemical weapons. OP nerve agents are divided into two groups: G agents and V agents. G agents contain a fluorine or cyanine as leaving group, whereas V agents contain a sulfur substituent leaving group.

Clinical Picture

OP poisoning shows typically the signs and symptoms of cholinergic crisis. Respiration is the most critical affected system. Severe poisoning causes respiratory depression, bronchosecretion, bronchospasm, and paralysis of respiratory muscles. Additional effects are miosis, increased secretions from glands, increased peristaltic activity, vomiting, general muscle weakness and twitching, hypothermia, bradycardia and hypotension, and convulsions followed by unconsciousness.

Toxicodynamic

Acetylcholinesterase (AChE) is one of the fastest-acting enzymes of the human body, which hydrolyzes the cholinergic transmitter acetylcholine (ACh), thereby inactivating its action on muscarinic or nicotinic receptors. Membrane-bound AChE is located at cholinergic synapses and neuromuscular junctions. Soluble AChE is present in the cerebrospinal fluid and in cholinergic nerve terminals. Nerve agents phosphorylate AChE at the active enzyme site, thereby inhibiting activity. As a consequence, ACh accumulates and overstimulates cholinergic receptors, leading to a cholinergic crisis. Antidotal therapy is directed either to competitively displace acetylcholine from the receptor (atropine) or to remove causally the nerve agent from its binding site (reactivation). To the later end, "reactivators" so-called oximes (e.g., obidoxime, pralidoxime) were introduced in causal therapy. This therapeutic strategy appears suitable in case of poisoning with several nerve agents (Sarin, VX). Unfortunately, however, AChE inhibited by several nerve agents can hardly be reactivated, e.g., tabun. Moreover, bound nerve agents undergo an "aging" process, where an alkyl or alkoxy group leaves the nerve agent AChE complex. The velocity of aging is dependent on the nerve agent and is extremely rapid in case of soman (aging half time about 2 min in humans). The "aged" complexes can no longer be reactivated. As a consequence, AChE reactivators as well as atropine should be given within minutes after exposure. Nevertheless, symptomatic treatment, e.g., artificial ventilation, may be necessary.

Biomonitoring, Bioanalytic, and Verification

To confirm clinical diagnosis based on typical signs and symptoms of cholinergic crisis, determination of red blood cell, AChE activity appears appropriate. This parameter can be determined even under field conditions or bedside within few minutes by the ChE-check mobile that is commercially available as certified as

medical products in Europe or in the United States by the Testmate®. Under several circumstances, however, ongoing treatment may be necessary, especially when active poison remains longer in the body than early administered antidotes. In such cases, aside from atropine, oxime treatment may be necessary for a longer period. To enable optimized patient-oriented application of oximes as long as needed, a laboratory test system, the so-called cholinesterase status, was established and is commercially available since early 2013. Apart from these clinically most relevant parameters, the analysis of intact nerve agent, its metabolites as well as protein and albumin adducts in body fluids are possible in special laboratories. However, for such analytical tasks, advanced techniques are necessary that are available only in a few laboratories.

Long-Term Effects

After exposure of organophosphate insecticides, an organophosphate-induced delayed neuropathy (OPIN) has been described. This clinical picture has not been observed in survivors of nerve agent poisoning. No reports about mutagenic, cancerogenic, or teratogenic effects after sarin, tabun, or VX poisoning have been published.

Vesicants

Sulfur mustard (bis(2-chloroethyl)sulfide, HD) was first synthesized in 1822 by Despretz. In World War I, it has been extensively used as chemical weapon and was called the "king of war gases." During World War II, nitrogen analogues such as ethylbis (2-chloroethyl)amine (HN-1), bis(2-chloroethyl)methylamine (mechlorethamine, HN-2), and tris(2-chloroethyl)amine (trichlormethine, HN-3) were synthesized in the United States. All these agents share their ability to induce skin blistering and were classified as "vesicants." Sulfur mustard is by far the most produced and stockpiled vesicant until today.

Clinical Picture (Short and Long Term)

Skin contact with sulfur mustard liquid or gas will produce blisters after a symptomless interval of several hours. Gaseous exposure affects more moist and hairy regions of the body as the genito-anal region, the chest, and axillae. The eyes are very susceptible. Even low vapor exposure results in ocular injury with severe blepharospasm. Inhalation of sulfur mustard vapor damages mainly the upper part of the respiratory tract. The trachea and bronchial epithelia become necrotic and detach from the wall (pseudomembranes). Besides this local effects, absorption of sulfur mustard results in systemic poisoning. Reproductive and developmental toxicity, gastrointestinal effects (vomiting, diarrhea), hematological effects (pancytopenia), and immunosuppression have been reported.

Toxicodynamic

Sulfur mustard is a lipophilic, alkylating substance with two reactive moieties. Sulfur mustard can easily penetrate the skin or other body surfaces and reacts with a huge variety of molecules. It can alkylate macromolecules and cross-link them.

The most important reaction is with the DNA. Sulfur mustard reacts predominantly with guanine at the N_7 position, which accounted for 61% of total DNA alkylation. Less likely are cross-links, 17% of alkylations involve two guanines (G-alkyl-G). However, cross-linked DNA strands are difficult to repair and cell division may result in DNA strand breaks, which are lethal lesions of the cell. Apoptotic cell death occurs with a delay of several hours.

This explains the late onset of clinical symptoms in organs characterized by high cell proliferation (e.g., skin). Despite a century of research and deeper insight in the pathophysiology of sulfur mustard poisoning, no causal treatment has been identified so far.

Late Effects

Sulfur mustard poisoning results in a variety of late effects. The most common late effects were found in the respiratory tract (42.5%), eyes (39%), and skin (24.5%).

The most disabling late effects after sulfur mustard inhalation are respiratory disorders, e.g., bronchiolitis obliterans, chronic obstructive pulmonary disease, asthmatoid bronchitis, and bronchial stenosis.

Late effects at the eyes are chronic keratoconjunctivitis. Only a few of exposed soldiers (0.5%) complain of a delayed type of ulcerative keratitis, which occurs several years after exposure and results in opacification of the cornea.

Balali-Mood et al. (2005) published a study on soldiers heavily exposed to sulfur mustard. The most important dermatological late effects are hyperpigmentation (55%), hypopigmentation (25%), erythematous papular rash (42.5%), dry skin (40%), multiple cherry angiomas (37.5%), and skin atrophy (27.5%).

As a DNA-damaging agent, it has been linked to several forms of cancer observed in workers or soldiers. Lung cancer (e.g., adenocarcinoma) has been reported in workers of sulfur mustard production facilities. Skin cancer (e.g., basalioma) may occur at exposed sites.

Biological Weapons

Definition

Biological weapons may be used for strategic or tactical reasons to intimidate, incapacitate, or kill an opponent, single individuals, or entire groups. The highest risk of a deliberate release of a biothreat agent currently arises from bioterrorism. Numerous species of highly infectious bacteria or viruses and various biological toxins have been misused as biological warfare agents in the past or are associated with an inherent risk to be misused due to their specific properties. Moreover, some species of fungi and parasites are listed as potential biothreat agents by some authors. Listing and current ranking of biothreat agents can be accessed at the websites of the American CDC, in the Chemical Weapons Convention, in the textbook of military medicine, or in the NATO handbook on the medical aspects of NBC defensive operations (AMedP-6(B)).

Among the biological warfare agents, biological toxins in contrast to live bacteria and viruses represent a group of noninfectious substances. Only toxins that can be utilized independently of their producer organisms are considered as autonomous biothreat agents and must be differentiated from toxins that are produced by the microorganisms during the course of infection and act as pathogenicity factors, such as the toxins of *Bacillus anthracis*. Biothreat toxins may cause Incapacitation, severe intoxication, or even death in exposed humans or animals. Early in history, various poisonous substances used to be employed not only for man's own survival but also to attack enemies. For the toxicologist, the risk assessment of toxin-derived "biological warfare agents" is principally the same as that of chemical warfare agents.

Characteristics of Biological Toxins

Toxins represent a subset of biothreat agents, which are also called mid-spectrum agents. They are noninfectious and do not reproduce in the host. The clinical manifestations of toxin-related diseases usually appear after a shorter latency period as compared to infectious agents. Naturally occurring biological toxins are synthesized by plants (curare, ricin), fungi (aflatoxins), amphibians (dart frog's batrachotoxin), bacteria (botulinum neurotoxin), or algae (paralytic shellfish poison) and are mostly part of the self-protection strategies of the producing organisms. The structures of biological toxins range from complexly assembled structures to simple bioregulator molecules: Complex AB toxins are produced by bacteria or plants. They consist of a binding (B) and an active (A) domain and interfere with internal cell functions. The binding subunit (B) binds to a cell surface receptor and enables the transport of the cytotoxic A-subunit into the cell. The sizes of AB toxins range from 25 kD to 200 kD (Table 2). Other toxins are non-peptide substances and rather bioregulator molecules. Their onset of action is immediate in contrast to AB toxins, which take effect with a latency period of hours, sometimes days. Their molar mass is smaller, ranging from 300 g/mol to 3000 g/mol (Table 2). They are also markedly stable under various environmental conditions, versus heat and pH alterations. They can even be synthesized in vitro (STX), which is not possible for the proteinaceous toxins. The trichothecene mycotoxins belong to the non-peptide substances and, moreover, are contact poisons. They gained notoriety as the "yellow rain" agent during the 1970s and 1980s in Cambodia and Laos, Southeast Asia, which is – for lack of unambiguous evidence – not without controversy.

Toxicological effects of biological toxins were studied mostly after alimentary uptake. However, more severe physiological consequences may result from exposure through a non-enteric route. Intentional exposure to toxins in aerosol and droplet clouds and after subcutaneous injection has occurred. Yet only few and inconsistent data is available with regard to the associated health effects. A variety of nonspecific clinical symptoms and multiorgan effects may develop depending on the way of exposure, ranging from acute emesis and diarrhea, nervous disorders,

Table 2 Characteristics of a selection of biological toxins without subtype differentiation

Origin	Name	Short	Listing	Main effect	Main pathophysiology	LD_{50}	Specific prophylaxis/ treatment	Size (kD)	Gold standard detection
Bacteria									
Clostridium botulinum	Botulinum neurotoxins	BoNT	AMedP-6(B) Cat A (CDC)	AB toxin, neurotoxic	Flaccid paralysis, botulism	0.001i.v; 1 p.o.	Antiserum, vaccine (limited)	160	Mouse bioassay
Clostridium perfringens	Epsilon toxin	–	AMedP-6(B) Cat B (CDC)	Pore-forming toxin, potassium, and fluid leakage from cells	Vasogenic brain edemas, indirect neuronal excitotoxicity	0.5	–	30	Mouse neutralization test
Staphylococcus aureus	Enterotoxins	SE	AMedP-6(B)	Emetic, toxic shock syndrome	Emesis, T-cell stimulation, cytokine release	0,02 inh	–	25	ELISA
Algae/plankton									
	Saxitoxin/ paralytic shellfish toxin	STX/ PST	AMedP-6(B) CWC	Neurotoxic, sodium channel blockage	Flaccid paralysis	6	–	0.3	Mass spectrometry
Plant									
Ricinus communis	Ricin	–	AMedP-6(B) CWC Cat B (CDC)	AB toxin, inhibition of protein synthesis, cytotoxic	Tachycardia, hypotension, seizures, multiorgan dysfunction	3i.v., >1000 p.o.	Vaccine in development	65	In vitro bioassay

(continued)

Table 2 (continued)

Origin	Name	Short	Listing	Main effect	Main pathophysiology	LD_{50}	Specific prophylaxis/ treatment	Size (kD)	Gold standard detection
Abrus sp.	Abrin	–		AB toxin, inhibition of protein synthesis, cytotoxic	Multiorgan dysfunction	>0.03i. v.;>10 p.o.	–	65	n.d.
Fungus									
Fusarium sp.	Trichothecene		AMedP-6(B)	Inhibition of protein synthesis					LC-MS/MS
	Deoxynivalenol	DON		Inhibition of protein synthesis	Emesis	10E7		0.3	LC-MS/MS
	T-2 mycotoxin	T-2		Inhibition of protein synthesis	Aleukia, cancerogenic	10E3	–	0.47	LC-MS/MS

Abbreviations: LD_{50} human LD_{50} (μg/kg), *i.v.* intravenously, *p.o.* per os, *inh.* inhaled

cardiovascular alterations, hemostatic derangements, skin toxicity, and multiorgan failure to chronic syndromes such as immunosuppression, weight loss, decreased reproductive capacity, and bone marrow damage.

Risk Assessment Aspects

Due to their relative ease of production and immense toxicity, some biological toxins are considered as potential biological warfare agents. The Centers for Disease Control (CDC, Atlanta, United States) provide the most widely used priority categorization of bioterrorism agents according to the risk to national security associated with them. Features determining the categorization are the ease of transmission/dissemination, the mortality rates, and the public health impact. The botulinum neurotoxins are classified as category A (highest priority). Ricin, staphylococcal enterotoxins, further clostridial toxins, and cholera toxin are classified as category B (second priority) agents. As listed in Table 2, biological toxins are also considered in the NATO handbook on the medical aspects of NBC defensive operations (AMedP-6 (B)) and most officially in the Chemical Weapons Convention.

In a military scenario, ricin and the botulinum neurotoxins are – besides the causative agents of anthrax or pneumonic plague – also considered as high-risk agents for bioterroristic or warfare activities. Risk-ranking respects the dimension of damage and the probability of an intentional event associated with the respective substance in a given scenario.

Low-dose pharmaceutical drugs containing botulinum neurotoxin (Botox) are commercially produced for the medical treatment of various neurological syndromes (Dysport®, Ipsen Biopharmaceuticals; Myobloc® Solstice Neurosciences; Botox®, Allergan). Moreover, in recent years, the cosmetics industry has established a fairly new market for botulinum neurotoxin due to its effect of wrinkle reduction. Every year, around 75 billion dollars are reaped with such products, which has given rise to large-scale non-licensed production of Botox drugs that are distributed via the internet. Illegal Botox production plants have settled in China, India, and the successor states of the former Soviet Union and might become a potential toxin source for bioterrorists. Ricin was researched for its ability to kill tumor cells during cancer treatment. However, pharmaceutical products have never emerged from such scientific approaches.

Risk Management

Biological Weapons Convention (BWC)

The BWC is an international agreement on the prohibition of the development, production, and stockpiling of bacteriological (biological) and toxin weapons and on their destruction. It was implemented in 1975 as a first multilateral disarmament agreement based upon the 1925 Geneva Protocol. It lacks the listing and ranking of

possible agents. So far, the BWC has 179 member states and 6 signatories. Eleven states have neither signed nor ratified the BWC. A major shortcoming of the BWC is its lack of a verification regime, which makes it difficult to prosecute noncompliance.

Chemical Weapons Convention (CWC)

Organization for the Prohibition of Chemical Weapons (OPCW)

Since 1997, the OPCW, located in The Hague, Netherlands, has been authorized to execute the controls and sanctions regarding the CWC as the official implementing body. Today, the organization comprises 193 member states and is directly responsible to the United Nations committee. OPCW received the Nobel Peace Prize in the year 2013.

To fulfill its tasks, the OPCW is comprised of several organs: the Technical Secretariat regulates administration, controls verification of international CWC implementation, and coordinates routine inspections. In return, decisions are made by the Executive Council and the Conference of the States Parties. They resolve questions of policy and matters arising between the States Parties on technical issues or on interpretations of the Convention.

Two of the biological toxins are listed in Annex B, Schedule 1, Numbers 7 (saxitoxin) and 8 (ricin).

National Regulations: Installation of Preparedness Standards

Laboratory Safety

As regulated in the CWC, the production, acquisition, and handling of quantities of more than 100 grams of a listed agent per year require permission. For the time being, only a few biological toxins are available in small amounts in the free market for research, analytical, or therapeutic issues.

Regarding safety at work on biological toxins in Germany, a national Committee on Biological Agents establishes or adapts the rules, which are officially released by the Federal Ministry of Labour and Social Affairs as Technical Rules for Biological Agents (TRBA). The most basic documents are the following TRBAs: “Protective Measures for Specific and Non-specific Activities involving Biological Agents in Laboratories” (TRBA 100) and “Basic Measures to be taken for Activities involving Biological Agents” (TRBA 500). Accordingly, handling of biological toxins is allowed in laboratories at containment level 1 (toxins) or a higher containment level corresponding to the risk group of an associated organism (e.g., level 2 for *Clostridium botulinum* strains). According to the international Globally Harmonized System of Classification and Labeling of Chemicals (GHS), tagging of vials containing biological toxins is required by use of a pictogram and a signal word (i.e., “Danger” or “Hazard”). Additionally, an individual material safety data sheet is required for each substance or mixture that mandatorily lists all hazard and precautionary statements.

Table 3 AEGL values (mg/m^3) for selected chemical warfare agents (Watson et al. 2006)

		Sarin (GB)	Tabun (GA)	Soman (GD)	Cyclosarin (GF)	VX
AEGL-1	10 min	0.00690	0.00690	0.00350	0.00350	0.00057
	30 min	0.00400	0.00400	0.00200	0.00200	0.00033
	1 h	0.00280	0.00280	0.00140	0.00140	0.00017
	4 h	0.00140	0.00140	0.00070	0.00070	0.00010
	8 h	0.00100	0.00100	0.00050	0.00050	0.00007
AEGL-2	10 min	0.08700	0.08700	0.04400	0.04400	0.00720
	30 min	0.05000	0.05000	0.02500	0.02500	0.00420
	1 h	0.03500	0.03500	0.01800	0.01800	0.00290
	4 h	0.01700	0.01700	0.00850	0.00850	0.00150
	8 h	0.01300	0.01300	0.00650	0.00650	0.00100
AEGL-3	10 min	0.38000	0.76000	0.38000	0.38000	0.02900
	30 min	0.19000	0.38000	0.19000	0.19000	0.01500
	1 h	0.13000	0.26000	0.13000	0.13000	0.01000
	4 h	0.07000	0.14000	0.07000	0.07000	0.00520
	8 h	0.05100	0.10000	0.05100	0.05100	0.00380

Risk Management

Besides international regulations to reduce the stockpiles of chemical warfare agents, national regulations are necessary to reduce health risks for the general population and emergency personnel. As the risk for terrorist attacks with chemical warfare agents or similar substances rises, toxicity estimates and exposure guidelines have been recently updated to ensure a more realistic national preparedness. In the United States, Acute Exposure Guideline Levels (AEGLs) have been developed and published (Watson et al. 2006). AEGLs were calculated for vapor exposure (10 min–8 h). AEGL-1 has been defined as a threshold where first mild symptoms are noticed, e.g., miosis for nerve agents. On the other hand, AEGL-3 vapor concentrations may induce severe life-threatening health effects. The published data (Table 3) can be used for planning and risk management to counteract terrorist attacks with chemical warfare agents.

Laboratory Standardization Approaches

Since 2012, an expert laboratory network has been constituted for the Establishment of Quality Assurance for the Detection of Biological Toxins of Potential Bioterrorism Risk (EQuATox), which since 2016 is continued in the Horizon 2020 funded network European program for the establishment of validated procedures for the detection and identification of biological toxins (EuroBioTox). Its goal is to build up a network of European laboratories that use equal standards for the detection and identification of biotoxins. The network is about to develop and validate improved analytical tools, reagents, reference materials, and standard operating procedures based on realistic incident scenarios. After comprehensive proficiency testings, best practice procedures will be determined and disseminated across Europe.

Pharmacy

The availability and development of antidotes against chemical warfare agents is a continuous challenge. For several chemical warfare agents, e.g., mustard, no specific antidote exists in spite of decades of research. In recent years, new technologies were developed, allowing a deeper insight into the mechanism of toxicity, and new approaches are under investigation possibly enabling improved wound healing. In other cases, e.g., nerve agents, new autoinjectors containing an oxime, atropine, and benzodiazepam are under development. As commercial interest in antidote development generally is very low, national financial support is crucial to sustain research efforts and to allow development of new devices, e.g., autoinjectors or new promising approaches to improve therapy.

During World War II, toxoid vaccines were investigated by the United States to protect researchers working on the production of biological warfare agents. Since then, further vaccines against biological toxins have been developed, among them the pentavalent PBT vaccine (CDC) against five serotypes of botulinum neurotoxin, the RiVax™ Ricin Toxin Vaccine (Soligenix), and a candidate vaccine against staphylococcal enterotoxin B (USAMRIID).

A very limited number of heterologous antitoxin products are available for the treatment of botulism (e.g., trivalent Botulismus-Antitoxin Behring, Novartis, heptavalent BAT® Emergent BioSolutions Inc. (FDA approved)). Besides the few specific treatment options, therapy relies on supportive measures and in most cases requires intensive care facilities.

A network of specific poison control centers is available throughout European countries. They are associated with local hospitals and store antitoxins and provide expertise regarding the treatment of intoxications.

Decontamination

Decontamination of body parts after exposure to chemical warfare agents or biological toxins is accomplished by cleaning with soap and water. Pharmaceutical products such as Reactive Skin Decontamination Lotion (RSDL) may be used for decontamination of skin surfaces contaminated with chemical warfare agents or biological toxins with skin absorption (trichothecene group) (Table 2). Wounds and lesions may be flushed with physiological solutions. For the decontamination of equipment, protein-denaturing dilutions of sodium or calcium hypochlorite may be used.

Cross-References

- ► Checklist: Toxicological Risk Assessment in Practice
- ► Data Mining in Toxicology
- ► Importance of Physicochemical and Physical Properties for Toxicological Risk Assessment
- ► Principles of Analytical Chemistry for Toxicology

▶ Risk Communication: Challenges for Toxicologists and Other Risk Experts
▶ Risk Management in Toxicological Disasters
▶ Toxicity Testing In Vitro: Regulatory Aspects
▶ Toxicological Risk Assessment

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Nanoparticles and Their Regulation

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Otto Creutzenberg

Contents

Abstract

Since the year 2000, nanotechnology has been an emerging factor in science, economy, and also daily life. Although nano size was well known in the past in chemistry (e.g., kolloids, catalysts), due to the huge number of engineered synthetic nanomaterials and their wide range of technical applications, a new broad economic field has come up. Regulatory bodies developed strategies to include nanomaterials into the existing legislation of chemicals, biocides, and food additives. This process was facilitated by the fact that principally not the chemistry changed from micro- to nanoscale but mainly physico-chemical properties (specific surface, solubility, agglomeration status, etc.). Thus, toxicological effects were not expected to be principally different; however, the evaluation had to be expanded from target organ effects (e.g., lungs, skin) to potential systemic effects. Due to the tiny size and an increased dissolution of nanoparticles, the toxicokinetics became a predominant additional endpoint. This summary is based on the technical guidelines, decisions, and laws issued by the main regulatory

O. Creutzenberg (✉)
Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany
e-mail: otto.creutzenberg@item.fraunhofer.de

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bodies such as the Organisation for Economic Co-operation and Development (OECD), European Chemicals Agency (ECHA), European Food Safety Authority (EFSA), European Medicines Agency (EMA), U.S. Food and Drug Administration (FDA), and U.S. Environmental Protection Agency (EPA). This chapter is presenting the regulatory status of nanomaterials being aware of the still ongoing process.

Keywords

Nanoparticles · Regulation · Agglomeration · In vivo · Dispersion · Translocation

Abbreviations

ANSES	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (French Agency for Food, Environmental and Occupational Health & Safety)
BAuA	Federal Institute for Occupational Safety and Health, Division 5 "Federal Office for Chemicals"
BPR	Biocidal Products Regulation
CNT	Carbon nanotubes
CoRAP	Community rolling action plan
DF4nano	Decision-making framework for the grouping and testing of nanomaterials grouping
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ENM	Engineered nanomaterials
EU	European Union
FDA	U.S. Food and Drug Administration
GD	Guidance document
IARC	International Agency for Research on Cancer
MoA	Mode of Action
MWCNT	Multi-walled carbon nanotubes
NBCDs	Non-biological complex drugs
NM	Nanomaterials
NP	Nanoparticle
OECD	Organisation for Economic Co-operation and Development
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SCCS	Scientific Committee on Consumer Safety
SWCNT	Single-walled carbon nanotubes
TDMA	*Titanium Dioxide*Manufacturers Association
TG	Technical guideline

TGP	Test Guidelines Programme
WPMN	Working Party on Manufactured Nanomaterials
USA	United States of America

Introduction

The nanotechnology industry has expanded enormously since the 1990s as scientists, engineers, and technologists have developed useful applications of nanomaterials in manufacturing, transportation, communications, energy production, waste treatment, consumer products, and medicine. The rapidly increasing number of engineered nanomaterials (ENM) raised the question how potential risks could be minimized and managed to allow a safe commercialization. There is a common understanding that existing legal frameworks sufficiently protect public health and the environment and could be used by policymakers to regulate ENMs, provided that nano-specific adaptations would be introduced.

Nanomaterials are solid, liquid, or gaseous substances typically between 1 and 100 nm in diameter or length. A single nanoparticle shows a "viral" size in between subatomic particles (protons, electrons) and the smallest microscopic ones seen through a conventional light microscope (e.g., red blood cells). By origin, they have natural or engineered sources. Nanoparticles are often highly reactive because of their large surface area-to-volume ratio and chemical reactions occur on surfaces (e.g., use as catalysts).

Many nanoscaled metal oxides are important high-volume chemicals, for example, titanium dioxide and zinc oxide nanoparticles (used as pigment dusts and sunscreens). Besides industrial and consumer applications nanomaterials are used in the medical field: for example, silver nanoparticles have antimicrobial properties and nanoscaled "carriers" can be used to administer pharmaceuticals selectively at body localizations with higher concentrations than achievable by intravenous administration. According to an estimate, the global market for products containing ENMs will be more than 3 billion € by 2020.

Regulation of Nanomaterials

Initial Considerations

Assessing the Risks of ENMs

ENMs may pose some risks to human health and the environment that are not well understood at this time. Exposure to ENMs can occur in many ways. The most direct forms of exposure can happen when ENMs are used in medicines, cosmetics, foods, or other consumer products. Exposure can also occur, however, when manufacturing, distributing, selling, disposing of, or recycling products containing ENMs. Nanomaterial waste products, which can be released to the environment at various stages of manufacture, use, and disposal, are another source of exposure to ENMs.

The toxicokinetic behavior of ENMs differs from their microscaled counterparts. Because of their tiny size, ENMs can enter the bloodstream, translocate through the body, accumulate in organs or tissues, cross the blood-brain barrier, and even penetrate into the cell nucleus.

How Should ENMs Be Regulated?

There is existing a controversial discussion:

Proponents of new regulations argue that ENMs are so different from existing substances and pose such far-reaching and poorly understood risks to public health and the environment that new forms of government oversight, such as regulations that address ENMs as a class, are needed. Opponents of new regulations argue that existing legal frameworks have been successfully applied to emerging technologies in the past (such as gene therapy and genetically modified organisms), so there is no need for new regulations tailored to ENMs. Opponents also point out that the heterogeneous nature of ENMs makes it difficult if not impossible to develop regulations for ENMs as a class.

Since the 1980s, the precautionary principle has played an important role in policy debates concerning climate change, chemical regulation, food safety, and other public health and environmental issues. A precaution is reasonable if it appropriately balances competing moral and social values, such as protecting public health and the environment, on the one hand, and promoting industry, agriculture, and the economy, on the other.

Policymakers should use existing laws to (1) regulate ENMs and the best available evidence to set regulation levels without creating new laws or an overarching system to regulate ENMs and (2) support additional research on the risks of ENMs (Resnik 2019).

Presently, there is no specific international regulation, no internationally agreed upon protocols or legal definitions for production, handling or labeling, testing toxicity, and evaluating the environmental impact of nanoparticles (NP). Medical standards related to ethics, environmental safety, and medical governance have been modified to cover the introduction of engineered nanomaterials (NM) into the biomedical field. Currently, the USA and the European Union (EU) have strong regulatory bodies and guideline legislation to control the potential risks of NMs. The European Commission has developed several pieces of EU legislation and technical guidance, with specific references to ENMs. This legislation has been employed inside EU countries to ensure conformity across legislative areas and to guarantee that an ENM in one sector will also be treated as such when it is used in another sector (Jeevanandam et al. 2018).

According to Jeevanandam et al. the toxicity of NMs depends on various factors:

- Dose and exposure time effect;
- Agglomeration and concentration effect. Increasing the NP concentration promotes agglomeration (microsized NP agglomerates);
- Particle size effect;

- Particle shape effect, for example, graphenes or MWCNTs;
- Surface area effect: the specific surface of a given particle mass increases with decreasing particle diameters;
- Crystal structure effect, for example, the two crystalline polymorphs of TiO_2 (rutile and anatase) show different toxicity;
- Surface functionalization effect.

Exposure Paths

The most relevant exposure path of powdrous nanomaterials at workplaces is the inhalation route during the production process because a majority of nanomaterials are dry powders and can easily build aerosols. Alternative exposure paths such as oral or dermal exposure are of lower importance and, however, can reach relevance for consumers (e.g., cosmetics, sunscreens, silver on surfaces of beverage cans). In fact, nanoscaled particles are capable of entering the body through skin pores, debilitated tissues, injection, and olfactory, respiratory, and intestinal tracts. Their entry may lead to various diversified adverse biological effects (Yah et al. 2011).

Chemicals: Present Regulatory Status of Nanomaterials

Regulatory Status at OECD

Together with the Test Guidelines Programme (TGP) of the Organisation for Economic Co-operation and Development (OECD), the OECD Working Party on Manufactured Nanomaterials (WPMN) has explored the need for adaptation of some of the existing OECD Test Guidelines (TGs) and Guidance Documents (GDs) as well as developing new TGs and GDs to specifically address NM issues. An overview of progress is provided in the TGP and WPMN, and information on supporting initiatives, regarding the development of TGs for nanomaterials addressing physical chemical properties, effects on biotic systems, environmental fate and behavior, and health effects. Three TGs were adopted, dealing specifically with additional questions concerning the proper testing of manufactured nanomaterials: a new TG318 "Dispersion Stability of Nanomaterials in Simulated Environmental Media," and adaptation of TG412 and TG413 on "Subacute Inhalation Toxicity: 28-Day Study/90-Day Study." The associated GD39 on "Inhalation Toxicity Testing" has also been revised. The TGP currently develops four new TGs and four GDs. One new TG and six GDs are developed in the WPMN. Six new proposals were submitted to the TGP in 2018. Furthermore, as TGs are accompanied by OECD harmonized templates (OHTs) for data collection, an outline of recently developed OHTs particularly relevant for NMs was also included (Rasmussen et al. 2019).

EU Definition of Nanomaterials

As a basis for a harmonized regulatory approach, the European Commission has developed a recommendation for a definition of the term "nanomaterial" for regulatory purposes:

"A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.

In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50%.

By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials." (EU Commission 2011).

Currently it is still a challenge to identify nanomaterials, in particular when integrated in products, that is, to measure particle size and size distribution. The identification is relevant for finding out whether the nano-specific provisions apply. Furthermore, since several regulations require hazard testing of nanomaterials, for example, before authorizing them for the use in consumer products, there is the need to confirm that available test methods and guidance are suitable for nanomaterials and, where relevant, to develop nano-specific tests. Moreover, to facilitate the testing of a potentially huge variety of different nanomaterials, a sound approach to group them is necessary. More specific data on nanomaterials are required to address regulatory needs, to account for the specificities of nanomaterials, and to assess their safety (Rauscher et al. 2017).

Regulatory Framework of European Union

The European Chemicals Agency (ECHA) is in charge of registration of chemicals. The EU's regulatory framework generally covers nanomaterials. Companies producing or importing nanoscaled chemicals in volumes more than 1 ton per annum need to register their nanomaterials. The Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation addresses all chemicals including nanomaterials.

Legislation on specific products (cosmetic products, novel foods, biocidal products, medical devices) addresses nanomaterials, including requirements for information on nanomaterials (labeling) and assessment of the safety of these materials.

As grounds for a regulatory approach, the European Commission has created a suggested definition of nanotechnology. Currently, it is still a challenge for regulators to recognize and describe nanomaterials, significantly when they have been incorporated into products. The identification is essential for finding out if the nano-specific rules apply. Moreover, since many policies mandate hazard evaluation of nanomaterials before authorizing them for use, regulators must confirm that available test procedures and guidance are compatible with nanomaterials and, if not, develop nano-specific tests (Smith 2018).

ECHA introduced nano-specific clarifications and new provisions in the REACH annexes coming into act by January 1, 2020:

- Requirement to identify and characterise nanoforms (size, shape, surface chemistry of particles of the nanoforms).
- *'Particle' means a minute piece of matter with defined physical boundaries; 'agglomerate' means a collection of weakly bound particles and 'aggregate' means a particle comprising of strongly bound or fused particles.*
- *A 'set of similar nanoforms' can be handled jointly for hazard assessment, exposure assessment, and risk assessment because of low variation within property boundaries.* (ECHA 2018).

Regulatory Framework of the USA

In the USA, the regulation of nanotechnology largely falls under the auspices of the Food and Drug Administration (FDA) due to safety issues surrounding its use in medicine and personal consumer products.

While the FDA has voiced its support for the use of nanotechnology in innovative new products under its purview, the agency has also said it seeks to establish clear regulatory guideline grounded in existing practices and the available science.

When evaluating food additives, the FDA looks to find little to no risk from the intended use of nanotechnology products. Medications, by contrast, are examined not just based on their risk profile but also their expected benefit. These varying legal standards show how different contexts could result in multiple regulatory outcomes, even if two products have the same degree of risk (Smith 2018).

The following FDA statements were presented in a fact sheet:

- FDA does not make a categorical judgment that nanotechnology is inherently safe or harmful but uses an adaptive and flexible approach.
- Particular approaches for each product area will vary according to the statutory authorities.
- FDA's nanotechnology regulatory science research portfolio focuses on understanding interactions of nanomaterials with biological systems; and on the adequacy of testing approaches for assessing safety, effectiveness, and quality of products containing nanomaterials.
- FDA's regulatory policy approach is consistent with relevant overarching U.S. government policy principles supporting innovation. (FDA 2018).

Due to these different processes of manufacturing, different forms occur termed as primary particle, agglomerates, and aggregates (Fig. 1). The respective definition for nanomaterials is as follows:

- "particle is a minute piece of matter with defined physical boundaries";
- "agglomerate means a collection of weakly bound particles or aggregates where the resulting external surface area are similar to the sum of the surface areas of the individual components";

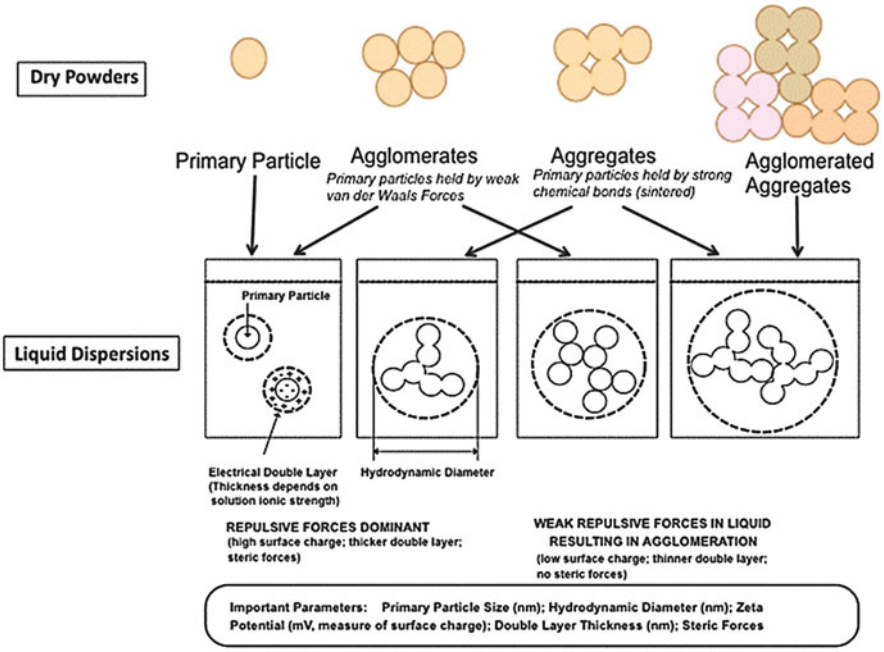

Fig. 1 Different forms of nanoparticles – schematic. (Adapted from Oberdörster 2010)

- "aggregate denotes a particle comprising strongly bound or fused particles" and the external surface can be smaller than the sum of the surface areas of the individual particles (Oberdörster 2010).

Approaches for Grouping and Categorizing Nanomaterials

A "Decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping)" was proposed by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) "Nano Task Force." It consists of three tiers to assign nanomaterials to four main groups, to perform sub-grouping within the main groups and to determine and refine specific information needs (Fig. 2). The DF4nanoGrouping covers all relevant aspects of a nanomaterial's life cycle and biological pathways, that is, intrinsic material and system-dependent properties, biopersistence, uptake and biodistribution, cellular and apical toxic effects (Fig. 3). The four main groups encompass

1. Soluble nanomaterials;
2. Biopersistent high aspect ratio nanomaterials;
3. Passive nanomaterials;
4. Active nanomaterials.

The DF4nanoGrouping aims to group nanomaterials by their specific mode of action that results in an apical toxic effect. This is eventually directed by a nanomaterial's

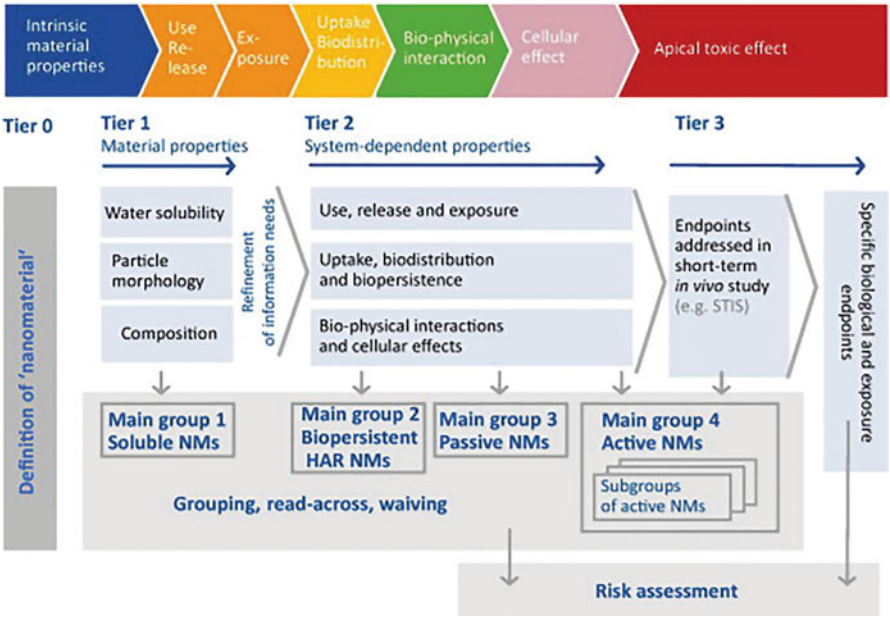

Fig. 2 The decision-making framework for the grouping and testing of nanomaterials. (Adapted from Arts et al. 2015)

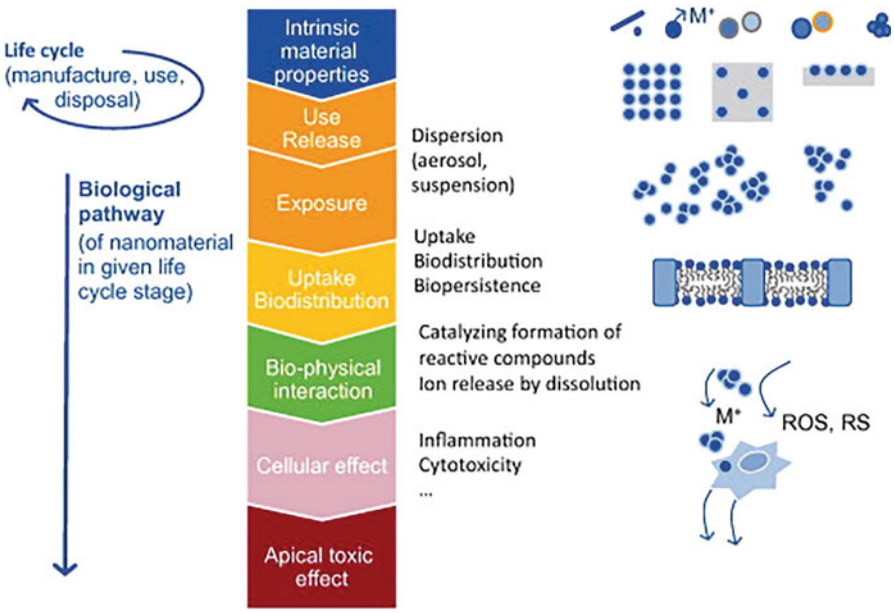

Fig. 3 Life cycle and biological pathway of nanomaterials. (Adapted from Landsiedel et al. 2010) M^+: metal ion; ROS: reactive oxygen species; RS: reactive species

intrinsic properties. The DF4nanoGrouping uses the "functionality" of nanomaterials for grouping rather than relying on intrinsic material properties alone. Such functionalities include system-dependent material properties (such as dissolution rate in biologically relevant media), bio-physical interactions, in vitro effects, and release/exposure (Arts et al. 2015).

Nanomedicine: Regulatory Status

Pharmaceutical manufacturing of nanomaterials involves two different approaches: top down (breakdown of a bulk material) and bottom up (starts with atomic or molecular species, then increasing in size).

The application of nanotechnology for medical purposes has been termed "nanomedicine" and is defined as the use of nanomaterials for nanodiagnosis, controlled drug delivery (nanotherapy), and regenerative medicine.

For regulatory purposes in Europe, the biological nanomedicines are under the framework set by European Medicines Agency (EMA). The industry frequently asks for scientific advice and a case-by-case is analyzed by the EMA.

Sometimes, the biological framework is the base for the **regulation** of the Non-Biological Complex Drugs (NBCDs) because they have some features in common: the structure cannot be fully characterized and the in vivo activity is dependent on the manufacturing process and, consequently, the comparability needs to establish throughout the life cycle, as happens to the biological nanomedicines.

For some NBCDs groups like liposomes, glatiramoids, and iron carbohydrate complexes, there are draft regulatory approaches, which help the regulatory bodies to create a final framework for the different NBCDs families (Schellekens et al. 2014). EMA issued principles addressing general issues regarding the complexity of the nanosystems and providing basic information for the pharmaceutical development, non-clinical and early clinical studies of block-copolymer micelle, "liposome-like," and nanoparticle iron (NPI), medicinal products created to affect pharmacokinetic, stability, and distribution of incorporated or conjugated active substances in vivo.

For regulatory purposes in the USA, the FDA oversees safety and efficacy of a broad spectrum of medical products (i.e., drugs, biologics, and devices) under the auspices of federal legislation and agency regulations and policy. Complex nanoscale products challenge this regulatory framework and illuminate its shortcomings for combination products. Fundamentally, definitions and supplemental FDA policies distinguish among three product areas based on whether the product has

- a chemical mode of action (drug),
  a drug is any chemically synthesized product intended for use in the "diagnosis, cure, mitigation, treatment, or prevention of disease";
  new drugs are those "not generally recognized" by qualified experts "as safe and effective for use under the conditions prescribed";

a biological product is a product that is "a virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, protein . . . or analogous product . . . applicable to the prevention, treatment, or cure of a disease or condition of human beings."

- a mechanical mode of action (device); a medical device is a product that is not a drug, meaning that it does not act through chemical action and is not dependent upon metabolism to achieve its primary intended purpose.
- a biological source.

The FDA published two guidance documents on nanotechnology in the context of medical products. One outlines considerations for industry when determining whether a product involves an application of nanotechnology, which indicates the need for sponsors to communicate nanotechnology status to the FDA as part of the product review process.

The other discusses a nanotechnology risk-based framework, specific requirements for conduct of non-clinical and clinical trials, manufacturing quality and controls, and special environmental considerations for drug and biologic products containing nanomaterials.

Cosmetics: Regulatory Status of Nanomaterials in Europe

Cosmetics are regulated as a specific class (EU Cosmetics Regulation – EC No 1223/2009) including nanomaterials. Nanomaterials are defined as "insoluble or biopersistant and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm." Cosmetic products containing nanomaterials must be notified to the Commission via the online Cosmetic Products Notification Portal at least six months prior to being placed on the market, including specific data relevant for risk assessment purposes (e.g., substance identification, physico-chemical properties, toxicological profile, and reasonably foreseeable exposure conditions). The Commission is using the Scientific Committee on Consumer Safety (SCCS) to perform a proper risk assessment of nanomaterials under concern. Cosmetics containing a nanomaterial require a "nano" label in the list of ingredients (SafeNano 2020).

Food/Food Packaging: Regulatory Status of Nanomaterials in Europe

Some nanomaterials can be detected in food products. These nanomaterials either occur as a by-product of the food additives' manufacturing process or they originate from natural sources. In contrast, nanomaterials are already used in food contact materials.

Materials that may contain **nanoparticles** and are used in food products can be divided into two groups: (1) inorganic compounds such as silicon dioxide, titanium dioxide, or silver and (2) organic compounds based on proteins, fats, or sugars.

Table 1 Synthetic nanomaterials used as food additives

Compound	E number	Usage
TiO_2	E 171	- whiten sweets, mozzarella, ice cream, or cake glaze
SiO_2	E 551	- drip aid for sauces, seasonings, or cappuccino powder - carrier for dyes or flavors - prevent turbidity of beverages
Silver	E 174	- Coloring of product surfaces (e.g., sugar, confectionery and bakery products)
Iron oxide	E 172	- color change
Gold	E 175	- coating of confectionery and chocolate or in liqueur

The most prominent representatives of this group are silicon dioxide (E 551), titanium dioxide (E 171), and silver (E 174); see Table 1. These approved food additives are not produced as nanomaterials and usually fulfill their purpose as significantly larger particles. During production, however, it cannot be prevented that a certain proportion of nano-sized particles are formed which can be considered as nanomaterials. The usage of such food additives is restricted to certain food products/groups and the maximal amount to be added is subject to official daily intake rates, for example, only 10 g/kg (corresponds to 10 per thousand) silicon dioxide in cheese are allowed.

Most commonly used food additives, which may contain certain percentage of nanomaterials © DaNa team.

Exemplary Cases of Nanoparticles Dominant in Commercialization

Classes of metals/metal oxides and carbonaceous materials are the most prominent players on the market. In the following, some substance-specific **regulation**s are presented. Metals/metal oxides exhibit their toxicity by means of the particle surface and, in addition, by dissolved metal ions. The degree of solubility of this material class in body fluids determines considerably the toxicological impact. Colloidal or nanosilver is used in biocidal products for disinfection and microbial inhibition on surfaces (fungi, viruses, algae, bacteria).

Titanium Dioxide (TiO_2)

TiO_2 is one of the high-volume chemicals that is commercialized in numerous nanoscaled variants. It exists in two important crystalline structures, rutile and anatas; often these forms are mixed and, in addition, made more hydrophilic or hydrophobic by coating the surface with functional groups. TiO_2 is regulated in various sectors, for example, as chemicals, as cosmetics, and as food additives.

Use as chemical (REACH classification): TiO_2 is an example showing the ongoing re-evaluation processes at ECHA; considered for decades as a non-carcinogen, new draft assessments are currently underway: in May 2016, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) submitted a proposal to ECHA for the classification of TiO_2 as a presumed

carcinogen (category 1B) by inhalation. After evaluating the proposal, ECHA's Committee for Risk Assessment (RAC) decided in September 2017 that there was insufficient evidence to classify TiO_2 in category 1B. Instead, the RAC was of the opinion that the evidence could only support a lower classification of TiO_2 as a suspected carcinogen (category 2) by inhalation only (TDMA 2020; ECHA 2017).

Cosmetic usage: Titanium dioxide is currently approved as a colorant and as a UV-filter in cosmetic products, including in the form of nanomaterial. Titanium dioxide (nano) is listed in entry 27a of Annex VI to Regulation (EC) No 1223/2009. It is allowed at a maximum concentration of 25% in ready for use preparation, except in applications that may lead to exposure of the end user's lungs by inhalation and subject to the characteristics listed in the entry (European Commission 2009).

Usage as food additive: TiO_2 is approved for use in food as the coloring additive E171, under the EU's General Food Law regulation. The safety of E171 is evaluated by the European Food Safety Authority (EFSA), which established in 2016 that data on E171 showed no health concerns for consumers.

In 2018, French authorities began a substance evaluation of TiO_2. Since then, the *Titanium Dioxide* Manufacturers Association (TDMA) has been working closely with the French ANSES and the ECHA to provide information and expertise responding to concerns and questions raised during this process.

On June 29, 2018, the EFSA reaffirmed the safety of E171, following a request for a scientific opinion from the European Commission (TDMA; EFSA 2019).

Silver

Silver nanoparticles release silver ions, which act as a biocidal substance. The biocidal activity of silver primarily was identified as an oligodynamic effect, that is, only very small portions of the active substance are needed for significant antimicrobial activity. Silver nanoparticles are incorporated, for example, into polymers to avoid microbial growth on their surface. The principal mode of action is described in Fig. 4. This mode of action (MoA) makes it difficult for regulators to judge the risk effects related to silver

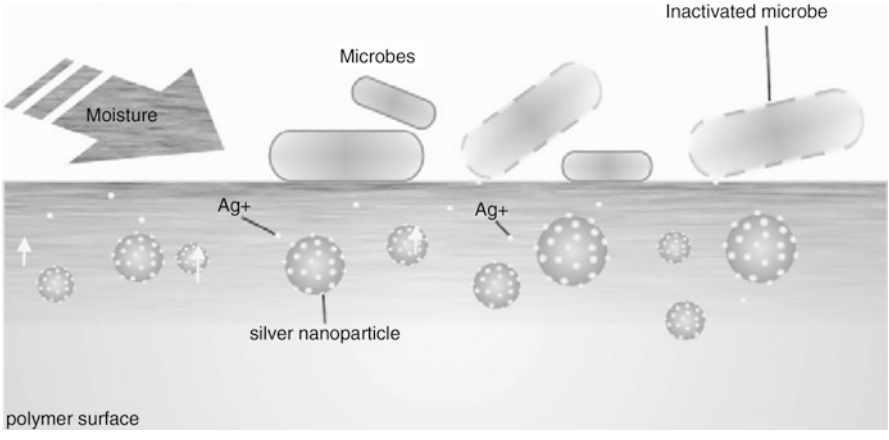

Fig. 4 Nanosilver effect on surfaces (depot effect). (Adapted from Schneider (2017)

nanoparticles. If materials are treated with silver to avoid the growth of germs (bacteria, fungi, yeast, virus, etc.) this application is inside the scope of the Biocidal Products Regulation BPR; 528/2012 EU (Schneider 2017).

Single-Walled (SWCNT) and Multi-Walled Carbon Nanotubes (MWCNT)

The global market of carbon nanotubes (thinner than 20 nm) is estimated to be around 200–250 tonnes (€30–40 million, mostly multi-walled carbon nanotubes) in 2009. The largest use is as a product imparting electrical conductivity to plastic materials, for example, in disk drive components or automotive plastic fuel lines and fenders (electrostatic coatings). Other uses include polymer additives, paints and coatings, fuel cells, electrodes, electrolytes and membranes in batteries, especially in miniature lithium batteries (BAuA 2018).

There are basically two shapes of CNT with different morphology: one variant with a tangled, low-density agglomerate form (SWCNT, MWCNT) and another one with a rigid, long-fiber (asbestos-like) morphology (MWCNT). The wall number and diameter determines the morphological outcome of MWCNT and a value between 20 nm and 45 nm makes the difference between the two morphological groups. All SWCNT and MWCNT with low wall number show the tangled form. MWCNT with higher wall number show asbestos-like fiber geometry and can correspondingly exert fiber-specific toxicity.

Experimental MWCNT samples (synthesized at lab scale) with diameters more than 50 nm (rigid type) were investigated in a chronic study in rats after intraperitoneal injection and showed a strong potential for the induction of mesotheliomas (Rittinghausen et al. 2014).

The International Agency for Research on Cancer (IARC) classified a certain group of multi-walled carbon nanotubes (MWCNTs) known as MWCNT-7 as "possibly carcinogenic to humans" (Group 2B) based on rodent studies that showed that MWCNT-7 caused peritoneal mesotheliomas in male and female rats after injection of CNTs into the peritoneum (intraperitoneal injection) and the scrotum (intrascrotal injection) and that inhalation promoted bronchioloalveolar adenoma and carcinoma in male mice. IARC found limited evidence for the two other types of MWCNTs with dimensions similar to MWCNT-7 and inadequate evidence for single-walled carbon nanotubes (SWCNTs) (IARC 2017).

Graphenes

Graphenes are two-dimensional allotropes composed of a single layer of carbon atoms, hexagonally arranged. Their experimental preparation succeeded in 2005 and the commercialization has started; however, regulation for this new material class is lagging behind.

Cross-References

- ▶ Biomolecules Versus Smaller Chemicals in Toxicology: ICH, EU, and US Recommendations
- ▶ Exposure Scenarios in Toxicology

- ▶ Importance of Physicochemical and Physical Properties for Toxicological Risk Assessment
- ▶ Microplastics: A Novel Suite of Environmental Contaminants but Present for Decades
- ▶ Read-Across Methodology in Toxicological Risk Assessment
- ▶ Risk Assessment of Food Additives
- ▶ Tobacco and Alternative Nicotine Products and Their Regulation

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Psychoactive Designer Drugs: Classes, Mechanisms, and Regulation

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Vincenzo Abbate, Claude Guillou, and Michael Schwenk

Contents

V. Abbate
Department of Analytical, Environmental and Forensic Sciences, King's College London, Franklin-Wilkins Building, London, UK
e-mail: vincenzo.abbate@kcl.ac.uk

C. Guillou
European Commission – Directorate General Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Ispra, VA, Italy
e-mail: Claude.Guillou@ec.europa.eu

M. Schwenk (✉)
Tübingen, Germany

Formerly: Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

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Abstract

Recreational drugs constitute a continuing problem worldwide that has recently been aggravated by the emergence of a growing number of new psychoactive designer drugs. The drugs of abuse problem is associated with risks of intoxications and is a menace to public health and safety. New designer drugs that emerge on the black market tend to be considered legal (also known as "legal highs") when labeled "not for human use" (e.g., "bath salts") and are not in the list of internationally controlled substances. The analogue-principle in its various forms aims at shutting down this open gate of abuse.

This chapter reviews the types of psychoactive designer drugs, their biochemical mechanisms of action, the difficulties of unequivocal identification of novel drugs and their metabolites, basic principles of regulation, and aspects of the analogue principle.

Keywords

Analogue principle · Analytical toxicology · Designer drug · Opiate · Psychoactive drug · Toxicology · EMCDDA · UNODC · DEA

Introduction

The term designer drug came up in the mid-1980s in connection with abuse of the then new, very potent synthetic opioid fentanil and some of its derivatives (Henderson 1988). The problem of ever new designer drugs has since increased and led to a cat and mouse game (Elliott 2011): Whenever a novel designer drug is identified and placed under control of the controlling agencies, new chemical analogues tend to appear on the illicit market for which no routine analytical method exists to detect and identify them and no science-based knowledge about their way of action and their pharmacological and toxicological effects. Such a cycle constitutes a big problem for policy and health authorities aside from the clinical and forensic toxicology experts.

Designer Drugs and Drug Design

Types of Designer Drugs

Designer drugs are found and misused or abused in various application areas, such as: (a) athlete doping (Cowan and Abbate 2020) which for instance involve novel beta-mimetics, synthetic anabolic steroids, absorbable peptide-analogues of growth hormone releasing hormone (GHRH), (b) performance supports such as phosphodiesterase type 5 inhibitors (PGE5i), (c) "fat burners" like the mitochondrial uncoupling agent dinitrophenol, (d) immediately acting poppers, and (e) the large

group of psychoactive drugs (Pourmand et al. 2018; Weinstein et al. 2017). Designer drugs often evolve as analogues of substances with known chemical structure and/or pharmacological activity. Such drugs are brought to the market, often via the "surface web" (internet) or darknet. They may or may not possess the pharmacological activity expected by the consumer. They are usually missing any quality control or safety testing such as routine preclinical- and clinical studies that are conducted during genuine drug development to evaluate effectiveness and toxicity. This situation is clearly of high concern for public health. It is increasingly challenging the law-enforcing institutions (Krasowski and Brown 2017), clinical and forensic toxicologists (Abbott and Smith 2015), and analytical chemists (Elliott 2011).

Drug Design

"Drug design" is a technical term that describes any targeted work of medicinal chemists in research and pharmaceutical development in synthesizing new pharmacologically active molecules. The concept of "drug design" came up in the last century, when structure-activity relationships became evident in connection with advances in the field of neurotransmitters and neurotransmission (Belleau 1970). Natural plant-derived pharmaceutical compounds and endogenous mediators were chemically modified in the search for pharmacologically active substances that would be superior to the then available therapeutic drugs with regard to efficiency, pharmacokinetics or safety profile. In such attempts, entirely new substance scaffolds were detected and developed, such as the synthetic opioids or the emerging designer benzodiazepines. Some substances were developed and marketed as prescription drugs, others introduced as research tools that helped to elucidate biochemical pathways or mechanisms of toxic action. Drug design today still works in a similar way, and includes modern methods, such as structure-activity relationship, QSAR models and synthesis-enforcing high throughput procedures.

Narcotic and Psychoactive Drug Classes

Ancient cultures have developed and used psychoactive drugs from local sources, such as plants or mushrooms. As a consequence of globalization, many previously culture-specific agents have spread all over the world. Some of them are now of medical importance as pharmaceuticals or precursors (e.g., morphine, cocaine, ephedrine).

Terms and Names

The meaning of the term "narcotic" partly differs among countries and jurisdiction, ranging between sleep-inducing, anesthesia-inducing, or stupefying substance that

may dull the senses, and opioid-like agents or any strongly addictive controlled substance. The term psychoactive or psychotropic is wider, encompassing any effect on neuronal brain function that alters the mind.

Types and Classes

Narcotics and psychoactive drugs of abuse can be classified in three groups, according to medically relevant psychic effect, chemical class, or biochemical mode of action, as shown in the three panels of Fig. 1. The left panel shows major medically defined groups and the associated characteristic psychic effects. The middle column lists some major chemical groups and substances. The brackets between the left and the middle panel provide a rough orientation for the typical assignment of chemical classes to pharmacological effects. The panel on the right specifies important receptor types that are targeted by neuroactive designer drugs.

The official lists released by drug-controlling agencies are governed primarily by chemical names according to the nomenclature of the International Union of Pure and Applied Chemistry (IUPAC, https://iupac.org), which describes the position of each atom and chemical group in the three-dimensional molecular structure. As an

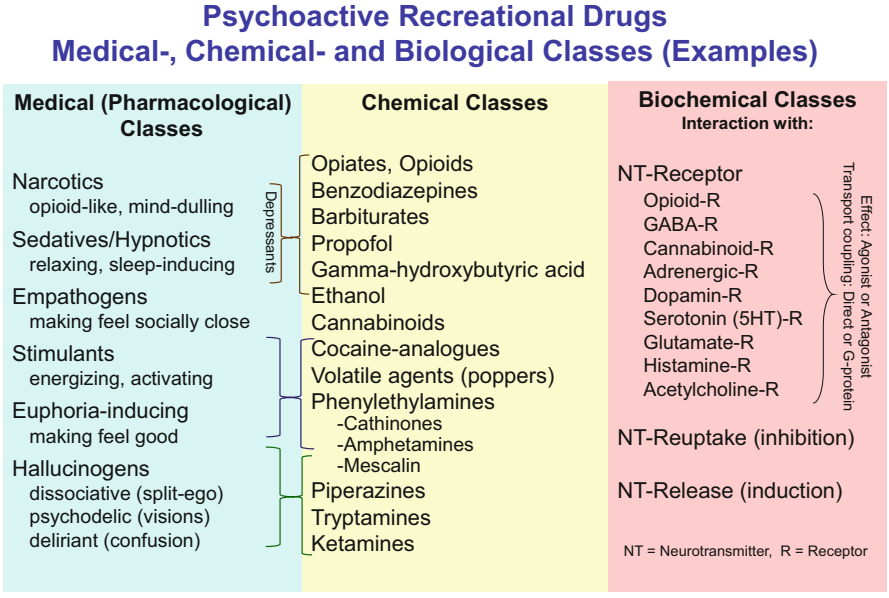

Fig. 1 Psychoactive Recreational Drugs. The three panels demonstrate how drugs can be classified according to medical, chemical and biochemical features. Narcotics and sedatives/hypnotics together form the majority of depressants. The brackets between the left and middle panel provide a simplified and incomplete assignment of chemical class to medical class; there exist overlaps and exceptions, e.g., mescalin (hallucinogen), belonging to the chemical group of phenylethylamines, that would typically be expected to be stimulants or emphatogens

example, carfentanil, a potent synthetic opioid, is now included in the list of scheduled substances with the IUPAC name "methyl 1-(2-phenylethyl)-4-[phenyl (propanoyl)amino]piperidine-4-carboxylate." Other chemical names for this substance can be found in several reference sources (e.g., in PubChem " methyl 1-(2-phenylethyl)-4-(*N*-propanoylanilino)piperidine-4-carboxylate"), which are also valid synonyms for naming carfentanil according to IUPAC naming rules. Despite the possible occurrence of several synonyms, IUPAC names are very important as a tool for chemical experts to unambiguously report and communicate the chemical structure of a substance. On the other hand, they are of little use for most people and for the non-chemists in particular. The latter tend to use International Nonproprietary Names (INN) for drugs (e.g., "carfentanil") or in some cases names that are composed of initials (often from the institution that synthesized, detected and/or recorded the substance), followed by a substance number. In addition, many designer drugs also have street names, sometimes even several other names after vendors or users habits. For each new designer substance, there may be a large number of names or terms to define it, which creates a further layer of burden to their already complex scenario.

Psychoactive drugs act upon the central nervous system (CNS), resulting in transient changes of consciousness, attention, perception, emotion, or mood. As recreational drugs, they are often taken purposefully to induce such changes. Many psychoactive drugs exhibit some (side-) effects on the peripheral neuronal system, thus affecting blood pressure, intestinal motility, or other functions.

A rapid uptake flux into the brain may be important for experiencing the full effect and having an immediate kick or "high." Intravenous injection, nasal administration ("snorting"), uptake via the oral mucosa or inhalation (smoking or "vaping") are among the most common routes for rapid uptake into the blood stream, while ingestion of a pill would lead to a protracted absorption and in some cases (partial) inactivation by means of first pass hepatic metabolism (biotransformation).

Parent Substances and Derivatives

A basic idea of psychoactive drug design concept is to use the scaffold of a known pharmacologically active drug and introduce chemical modifications. Increasing lipophilicity of these groups may improve permeability through the blood-brain barrier (BBB) and thus enhance the specificity of a drug to the central nervous system (CNS), compared to its effects on the peripheral neuronal system. The length of a lipophilic side chain, introduction of heterocycles, or introduction of halogen atoms belong to the more common modes of searching for derivatives that specifically act in the CNS, for instance, due to their increased BBB-permeability, or given the more favorable target receptor interactions. The synthesized product will then get purified, and in a pharmacological context, the most active chiral (if any) form may be isolated, however, racemic mixtures are found on black- and street markets due to the generally cheaper and easier synthetic routes for racemic substances. The

interaction with the biological target, very often a membrane-receptor that is associated with a membrane-transporter, may be stronger, weaker, inverse, or entirely absent, compared to the parent substance. A wide spectrum of effects is for example seen with the various noradrenalin-related drugs, some exhibiting preferentially CNS-effects (methamphetamine, cathinones), others preferentially acting as peripheral adrenalin mimetics or peripheral beta-receptor blocking agents.

It is not uncommon that minor chemical modifications lead to a substance that partly or fully interacts with other than the expected target in the CNS ("off-site" effects). An example is mescaline, which is primarily a hallucinogen (serotoninergic), in contrast to many other phenylethylamines that are CNS stimulants (noradrenergic and/or dopaminergic).

Finally, effect, strength, and toxicity profile of a psychoactive drug may be co-determined by minor but potent synthetic impurities.

Novel Psychoactive Substances

Novel (or New) psychoactive substances (NPS) are a broad and heterogenous group of substances of abuse that are presently not (yet) controlled by international conventions and pose a public health threat (Abbate et al. 2018; UNODC 2019; Fattore and Weinstein 2019).

Some NPS have formerly been developed as research drugs, e.g., as diagnostic tools to study in vitro neurobiological pathways. Others were synthesized in academic institutes or pharmaceutical industries, but later not marketed, e.g., due to insufficient therapeutic activity or safety concerns. A third group constitutes a category of entirely new substances, more recently synthesized on the basis of known scaffold molecules, which get slightly modified by including additional chemical residues. Some NPS are well-known recreational drugs, for which pharmacological, forensic, and toxicological literature exists; however, for many other substances, there exists no scientific literature or information, which poses high concerns for the public safety.

Synthetic cannabinoids and synthetic cathinones are the largest groups of NPS that were seized and/or reported for the first time to regulatory bodies in the past decades; however, many other derivatives of other chemical groups and subclasses also exist. Figure 2 shows chemical structures of nine selected NPS and the chemical class to which they can be assigned. The derivatives of morphine (opiates) have a long history that started with heroin more than 100 years ago. Later, the fully synthetic compound fentanil and some of its derivatives were identified, as well as the non-fentanil synthetic opioids.

Figure 3 shows in the upper row the parent substances that are used in different medical treatments (country-dependent, e.g., morphine for pain reduction or control, fentanil for anesthesia and pain reduction, methadone as substitution therapy); the lower row shows structures of analogues (heroin, carfentanil, U-47700) that are abused as recreational drugs.

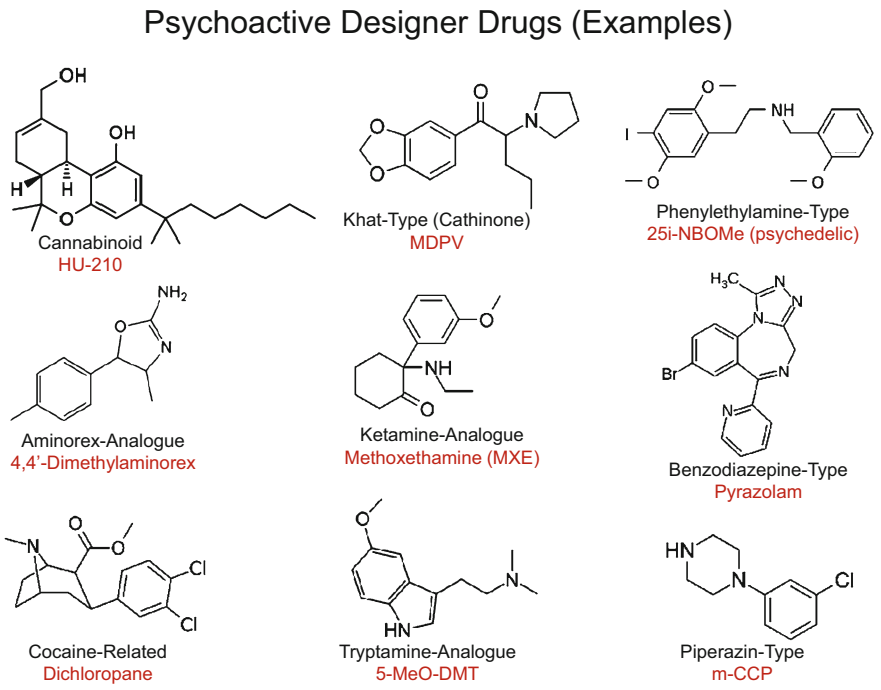

Fig. 2 Psychoactive Designer Drugs (Examples). The figure shows chemical structures of substances belonging to different psychoactive designer drug groups. Substance names are in red, names of the groups to which they belong are in black

Carfentanil has a very high potency. It is approved as veterinary pharmaceutical to tranquillize large animals, but not approved for human use. Carfentanil has recently been responsible for many fatalities due to overdoses despite the low dose that may have been used (Wilcoxon et al. 2018; Delcher et al. 2020). Some derivatives such as the opiate naloxone inhibit the opioid receptor. Naloxone is medically used as fast-acting antidote to reverse an opioid intoxication or opioid-induced respiratory depression.

Actions at the Neuronal Synapse

The Normal Neurotransmission Process

Psychoactive drugs have in common that they interfere with signal-transmission from the terminal of one neuron to a connected neuron. In the various brain areas, neurons fulfill specific functions, interconnected with the other areas. The information transfer and communication between neurons is via narrow gaps, the synapses, where arrival of an action potential in a presynaptic neuron leads to neurotransmitter release into the synaptic space. The various types of

neurotransmitter substances include small amino acid-derived molecules (e.g., noradrenalin), peptides (e.g., endorphins), lipid-derived substances (endocannabinoids), acetylcholine, and others. Binding to the respective membrane receptor on the postsynaptic neuron opens an ion channel, either directly (ionotropic) or via G-protein-mediated signaling (metabotropic) and thus elicits an action potential. Most neurotransmissions are directed from the presynaptic to the postsynaptic membrane. But there are exceptions. Thus, the endogenous cannabinoid system works in the opposite direction, endocannabinoids being released at the postsynaptic side, binding to a cannabinoid-receptor on the presynaptic membrane and then inhibiting or modulating the release of the principal neurotransmitter of this synapse.

Knowledge about the involved neurotransmitters, receptors and signal transduction pathways can be gained experimentally by a combination of in vitro tests with cultured neurons and isolated receptors, plus in vivo studies in lab animals using brain imaging methods, measurement of electric activities, or analysis of neurotransmitter patterns in various areas of the brain.

Interaction with Neurotransmitter Receptors

Neurotransmitter receptors on neuronal terminals tend to be quite specific for binding "their" (endogenous) neurotransmitter, though there are exceptions, such as the trace amine-associated receptors (TAARs) that accept several different neurotransmitters. Psychoactive drugs typically bind as ligands to neurotransmitter receptors and thus mimic or sometimes antagonize neurotransmitter-effects. An example are opiates/opioids. The endogenous ligands of opiate receptors are the endorphins, which are peptides. Surprisingly, the structurally unrelated substance morphine and analogues such as codeine or heroin also bind these receptors. Moreover, other classes of structurally unrelated fully synthetic substances like fentanil and its derivatives as well as non-fentanil opioids also bind to opioid receptors (Fig. 3).

Most neurotransmitter receptors exist in multiple subtypes, as exemplified with receptors for endorphin (kappa-, mu-, delta-, and more), serotonin (5-HT-1 to 5-HT-7), adrenalin (alpha1, alpha2, beta1, beta 2, beta3), dopamine (D_1 and D_2 families and their subtypes), endocannabinoids (CB_1, CB_2), or the TAAR families. Receptor subtypes tend to differ with regard to their distribution on neurons or brain areas. Some subtypes mediate identical, others different or even opposite effects, some are subject to up- or downregulation by neuronal feedback mechanisms. The differential location within the network of brain neurons and the specific biochemical interactions of recreational drugs with the receptor subtypes will determine the total psychoactive effect.

Interaction with Neurotransmitter Transport

Some psychoactive substances act indirectly by inhibiting the reuptake transporter that shuts off a neurotransmission signal by shuffling the neurotransmitter from the

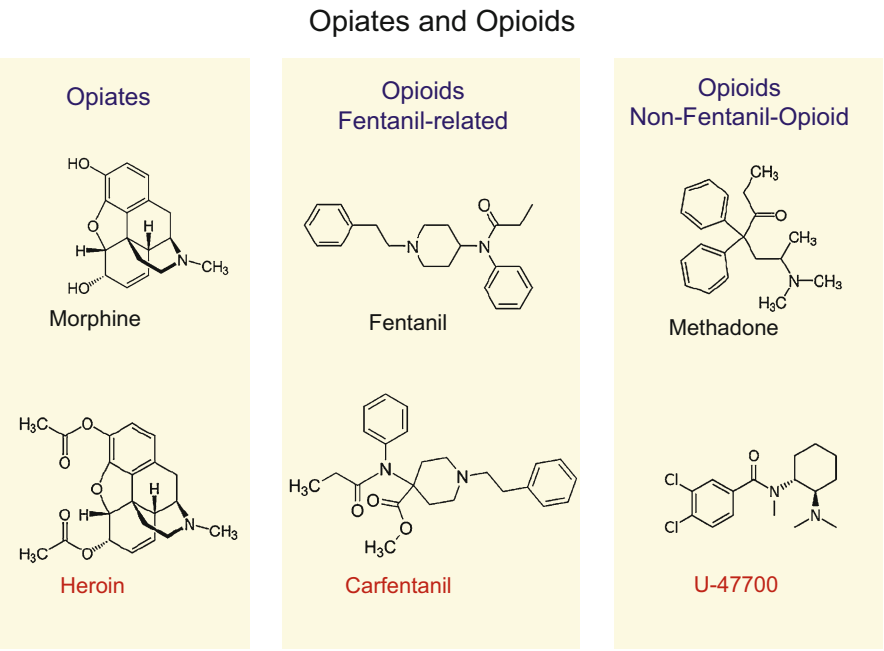

Fig. 3 Opiates and Opioids. The upper row shows chemical structures of three different classes of parent molecules that interact with the opioid-receptor. Opiates include herbal morphine or thebaine and semi-synthetic analogues derived thereof, like heroin. Opioids are synthetic compounds. Shown are fentanil-analgues and non-fentanil structures. The structures in the lower row are examples for respective analogues. Carfentanil is an extremely potent drug, U-47700 is a non-fentanil novel opioid, both found in the drug scene

synaptic space back into the presynaptic neuron. This inhibition leads to increased and prolonged presence of the neurotransmitter in the synaptic space and thus to a prolonged and increased signal. Another indirect mechanisms of action is seen with psychoactive drugs that are taken up into synaptic vesicles, causing displacement of the neurotransmitter into the synaptic space and thus activation of the postsynaptic neuron, even in the absence of a signal.

Interaction with Neurotransmitter Degradation

Other types of psychoactive drugs interfere with synthesis or degradation of neurotransmitter molecules. Examples include monoaminoxidase (MAO) inhibitors, a group of therapeutic drugs used to treat depression disorders.

Many psychoactive substances interact with more than one receptor type and affect several neurotransmitter pathways, thus producing the substance specific changes of brain function. The TAAR pathways (Freyberg and Saavedra 2020) are often involved (Fig. 4).

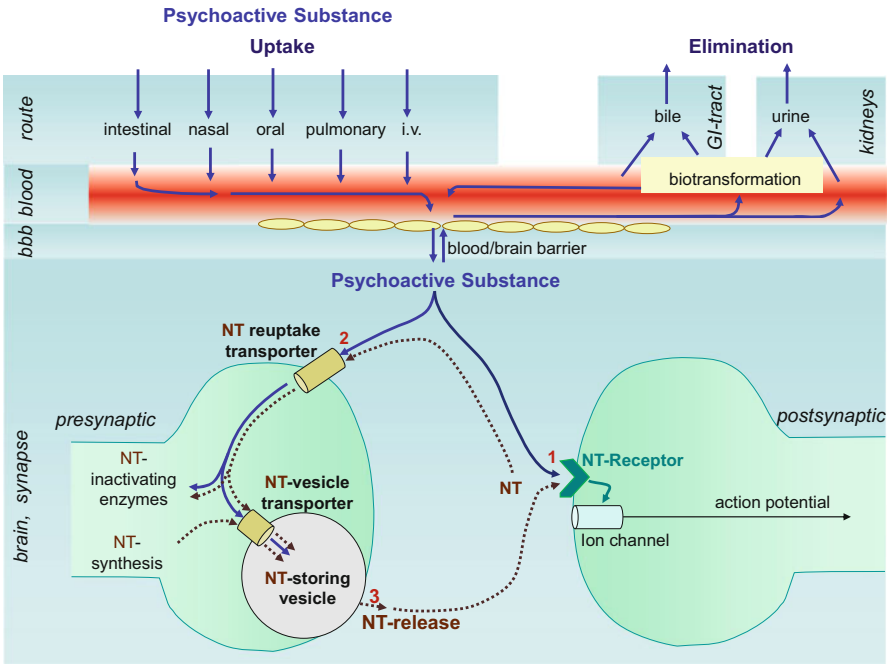

Fig. 4 Schematic drawing showing psychoactive substance (PS) disposition, and interaction with neurotransmitters (NT) in synapses (examples). PS may be taken up into the body via different routes. Once in the blood, a high lipophilicity tends to favor uptake across the blood-brain barrier (BBB) into the brain. Directly acting PS bind as agonist to the neurotransmitter NT-receptor (1), usually on the postsynaptic membrane, inducing via opening of ion channels an action potential. PS may also act indirectly either by inhibiting reuptake of the NT into the neuron (2), or displacing NT from NT-storing vesicles, thus inducing NT-release (3). Interactions with local NT synthesis or breakdown may also be involved. Main routes of PS elimination are via the liver and bile into the gastrointestinal tract (GI tract) or via the kidneys into urine, either unchanged or following biotransformation

Metabolism and Elimination

The metabolic inactivation of many endogenous neurotransmitters occurs rapidly by enzymes in or around the synapse. Some psychoactive drugs interfere with this breakdown.

The psychoactive drug molecules are usually subject to biotransformation primarily in the liver. This produces less active or inactive metabolites, which are then eliminated via bile or kidneys. But caution is required, since some metabolites may still be pharmacologically active. Examples are morphine-conjugates or certain phase I metabolites of synthetic cannabinoids.

In the analysis of psychoactive drugs in human body fluids of intoxicated persons or postmortem, it is of utmost importance to search not only for the parent substance but also to detect metabolites. Depending on the biological fluid used for the analytical investigation, metabolites may be present at concentrations exceeding those of the

parent drug. The full analytical pattern of parent drug plus metabolites provides a more complete picture to the clinical and forensic toxicologist with regard to the total amount of consumed drug or the time that elapsed since the most recent drug intake.

Toxicology and Clinical Toxicology

Acute Intoxication

Acute drug intoxications and fatalities are common when abusing (particularly the unregulated) psychoactive substances. One of the reasons is that drug consumption may cause a transient euphoria, and when the intensity of the effect subsides, the consumer takes further drug doses, until a toxic or fatal level is unexpectedly reached. This is often aggravated by the simultaneous intake of several different types of drug, some stimulating, some sedating ("polydrug" use), or when the agent has a higher potency, than expected by the consumer.

Thus, intake of propofol in connection with agents such as cathinones or amphetamines that affect intermediate metabolism may cause severe imbalances of energy metabolism. Fentanil and its derivatives, notably the very potent carfentanil today causes many intoxications (Wilcoxon et al. 2018; Delcher et al. 2020), with respiratory arrest, often leading to death. Amphetamines activate the cardiovascular system and produce hypertension often leading to cardiovascular emergencies.

When confronted with an intoxicated patient, the clinical toxicologist will initiate a general supportive therapy and general biochemical and drug-analyses if possible. Once the consumed substance is identified, this may help to use specific therapy such as an antidote or antibody.

A big problem arises from designer drugs, for which no toxicological profile, no forensic literature, and no specific therapeutic regimen exists. Given the low dose needed to cause intoxication for some novel potent derivative substances, often times the analytical toxicology of these substances is highly complex and requires sensitive and specific analytical instrumentations. Often times these are not available in clinical settings. Furthermore, in the absence of a patient anamnesis, a complete analytical investigation to identify the substances responsible for the intoxication, requires time-consuming analytical investigations carried out by highly trained personnel. The clinicians have thus to rely on common observed patterns of toxicity for the rapid clinical management of the intoxication. This again is difficult in the case of a new designer drug or polydrug use due to complex clinical manifestations.

Dose-Response Relationships

With increasing doses, the CNS-effects of psychoactive drugs tend to shift, as exemplified by the well-known sequence of ethanol-effects. These evolve with rising doses from initial sedation towards excitation, aggressiveness, coma, delirium, and sometimes death. The sequence may vary at the inter-individual level and depends on factors such as preceding stress, co-medication, age. In drug tolerance, the desired pharmacological response will require increased dosing, cause physical dependency, and increase the risk of acute toxicological emergencies.

Chronic Toxicity and Dependency

Prolonged abuse of metamphetamine-like substances may cause severe paranoid psychosis. Cannabinoids have a deleterious effect on the still developing brain of teenagers, some hallucinogens produce long-lasting flash-back experiences. Drug consumption during pregnancy is a common risk for the developing fetus and the normal development of neuronal networks in their brain. Babies with prenatal exposure to methamphetamine were reported to suffer from various deficit. This aspect of designer drug consumption will have long-lasting consequences for families and society and is alarming for public health institutions.

The variants of brain deteriorating effects are known for traditional recreational drugs, but the risks are unknown for novel designer drugs. Moreover, even impurities may exhibit potent neurotoxic effects; an example is the substance MPTP ("1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine") that occurred as a contaminant of the synthetic opioid-mimetic MPPP ("1-methyl-4-phenyl-4-propionoxypiperidine"). MPTP sadly induces Parkinson's-disease, after it is converted in the brain by monoamine oxidase to the neurotoxic agent MPP^+. MPTP has since been used as a tool to study the pathogenesis of Parkinson disease (Langston 2017).

Central Role of Analytics

Drug identification is a pillar in drug control. Seized samples, often powders, are brought to the specialized analytical lab. The material is checked with regard to visual appearance, color, smell, and consistency, analyzed with available immunochemical tests, and when required subject to detailed chemical analysis. When neither literature nor catalogues of analytical chemistry help to identify the analytical signals, a detailed procedure employing a full spectrum of methods, such as hyphenated chromatography-mass spectrometry and NMR may lead to the absolute structure elucidation and identification.

The pre-analytical procedures and sample clean-up are often simpler in urine than in blood. Urine has, however, the disadvantage that it tends to contain metabolites of the drug, rather than the original drug. To circumvent this problem, conjugate-metabolites can be subjected to chemical or enzymatic hydrolysis to release the scaffold for analytical detection. Nevertheless, the released substance is not identical to the consumed drug, when it has undergone hepatic phase-1 biotransformation. Analysis in blood samples provides the problem of multiple background substances (matrix components) from which the analyte has to be separated. If separation from background noise is successful, the chance for detecting the original drug may be greater than in urine samples, provided that not too much time has elapsed since drug intake.

Another analytical challenge is associated with the analyte itself. Many recreational drugs and designer drugs occur in enantiomeric forms, all having the same basic structure, of which only one chiral form may exhibit the psychoactive effect.

Smuggled drugs (e.g., as "black cocaine") have sometimes been mixed with agents to render difficult their detection in conventional assays or by sniffing dogs. These agents can get removed with specific methods by the dealer to make the drug "clean"

again. Similarly, when novel designer drugs are laced with prescription therapeutics, standard test methods may cause misleading results. Often a very detailed analytical procedure is required for an identification (Lobo Vicente et al. 2016). Communication and exchange between the analytical centers is important (Guillou et al. 2018).

Aspects of Regulation, Control and Health Protection

Drug regulation and the delineation of pharmaceutical drugs against recreational drugs have a long history. A milestone of regulation was around 1920, when many countries regulated the problems associated with opiates and the then new opiate-derivative heroin in "dangerous drug acts." Amendments were later made according to upcoming new types of drugs and international conventions followed.

It is inherently difficult to find a widely accepted regulation between full liberalization and full prohibition of recreational drugs, and the attitudes and rules are not entirely static, as demonstrated by the transient alcohol prohibition in the USA in the 1920s, the part-liberalization of cannabis starting in various countries around the year 2015, or the withdrawal in some countries of formerly available pharmacologically used agents such as the stimulating substance prolintane from markets. Scientific interest and drug development need a liberal regulation, when they are pushed too much towards prohibition, this may negatively affect a societies' scientific expertise and at the same time even enhance the danger of clandestine synthesis somewhere in the world.

National and International Efforts

In 1961, a number of international conventions were replaced by an updated, combining UN-convention "Single convention on narcotic drugs" that regulated mainly opioids, cocaine, and cannabis. In 1971 followed the "Convention on psychotropic substances," that included many other agents, such as hallucinogens. In 1988 followed the United Nations convention against illicit traffic in narcotic drugs and psychotropic substances, with respective lists. Relevant chemical precursor substances are also placed under control such as naphthoylindole, a precursor of designer cannabinoids or thebaine, a precursor for opiates (CND 2020).

Though the principles of regulation are adopted in national laws to the United Nations conventions, national laws tend to include country-specific aspects. At international level, agencies such as DEA in the USA, EMCDDA in Europa, and UNODC of the United Nations are well organized, cooperating institutions. The principles of the 1971 convention are basically still valid. Health aspects play a significant role including avoidance of addiction, public health protection, and the availability of important medicines (Fig. 5).

While many traditional recreational drugs of abuse are still in use and constitute an on-going dilemma, the ever-increasing number of new designer drugs poses additional new problems for the controlling agencies and health protection institutions. Illicit designer drug producers have access to the official lists of

Psychotropic Substances
UN-Classification Types (1971)

Schedule	Risk of Abuse	Threat to Public Health	Therapeutic Value	Level of Control
I	Very high	Very serious	Absent - little	Very strict
II	Risk	Serious	Low - moderate	Less strict
III	Risk	Serious	Moderate - high	Less strict
IV	Risk	Minor	High	Less strict

Fig. 5 United Nation Classification system (simplified table). Depicted are the schedules for psychotropic substances, and the respective criteria

NPS. With the help of published synthesis methods, they can reproduce substances or synthesize new derivatives that are not yet listed as controlled substances. For the controlling agencies, it tends to be a long way from unequivocal substance identification until the final international consensus is found to place the substance in the list of controlled substances. Nevertheless, this eventually happens. Examples are the substances mephedrone, BZP (1-benzylpiperazine) and MDPV (3,4-methylenedioxypyrovalerone), the synthetic cannabinoids JWH-018 and AM-2201, that moved from the status of NPS to scheduled International control in 2015. More recently, several other synthetic cannabinoids (AB-CHMINACA, ADB-CHMINACA, 5F-MDMB-PINACA, CUMYL-4CN-BINACA) and fentanil analogues including furanylfentanyl, acryloylfentanyl, 4-FIBF (4-fluoroisobutyrylfentanyl), THF-F (tetrahydrofuranylfentanyl), carfentanil, cyclopropylfentanyl, methoxyacetylfentanyl were then added to the list of substances of the Convention on Psychotropic Substances and to that of the Single Convention on Narcotic Drugs respectively.

The Analogue Principle

Types and Advantages

Regulation often applies the analogy principle to make NPS illegal in the absence of precise knowledge about their character, even before they come to the market. International, national, and state agencies use different wording, not least because a too simple definition of “analogue” may inadvertently include natural substances present in coffee and chocolate or alcohol, and any wording tends to still have gaps. In accordance with the classes in Fig. 1, the analogy principle is usually applied in any of three ways: by type of pharmacological class, chemical

class, and biochemical mechanism of action. A newly seized substance, for example, may be identified in the forensic analysis as a phenylethylamine derivative, but it is initially not known, what kind of psychoactive effect it bears. Therefore, it is hoped that receptor binding studies provide information about the mode of biochemical interaction with major neurotransmitter systems such as adrenergic, serotonergic, dopaminergic, etc. But even when specific receptor-binding studies give positive results, it is possible, that no signal transduction via, e.g., G-proteins to open a metabotropic pathway occurs. This detail must be studied in additional experiments. In conclusion, it may become very difficult to provide a final proof for analogy. In practice, the problem may be even more difficult to solve, when the seized material contained more than one phenylethylamine-analogues. This is a challenge for clinical and forensic toxicology, and notably for chemical analytics.

The multidimensionality of the analogue problem is reflected by the very detailed wording used in regulations, such as the 2019 Florida statutes (Florida 2019) first paragraph of the section on synthetic cannabinoids:

> 190. Synthetic Cannabinoids.—Unless specifically excepted or unless listed in another schedule or contained within a pharmaceutical product approved by the United States Food and Drug Administration, any material, compound, mixture, or preparation that contains any quantity of a synthetic cannabinoid found to be in any of the following chemical class descriptions, or homologues, nitrogen-heterocyclic analogs, isomers (including optical, positional, or geometric), esters, ethers, salts, and salts of homologues, nitrogen-heterocyclic analogs, isomers, esters, or ethers, whenever the existence of such homologues, nitrogen-heterocyclic analogs, isomers, esters, ethers, salts, and salts of isomers, esters, or ethers is possible within the specific chemical class or designation. Since nomenclature of these synthetically produced cannabinoids is not internationally standardized and may continually evolve, these structures or the compounds of these structures shall be included under this subparagraph, regardless of their specific numerical designation of atomic positions covered, if it can be determined through a recognized method of scientific testing or analysis that the substance contains properties that fit within one or more of the following categories:...

Measures for Health Protection and Control

The international community and most countries have developed many different activities and concepts that are suited to help drug addicted people, control, and avoid drug use. Here are some examples:

- Support for individuals with acute drug toxicity, e.g., Euro-DEN (Wood et al. 2016)
- Seizure-Activities
- Control of precursor substances
- Prediction of designer drugs (Carlsson et al. 2016, 2018)
- Shutdown of internet/darknet platforms
- Early warning systems, national, international

- Analysis in wastewater samples to monitor the broad population consumption
- Drug checking campaigns in party settings (voluntary, non-voluntary)
- Support for drug analyzing toxicology-centers (Guillou 2017)
- Support for clinical toxicology centers, specialized on intoxication treatments
- Abuse-resistant prescription drugs (Coleman et al. 2005), e.g., inapt for injection or nasal application
- Injection devises free of charge
- Methadone (or similar) substitution programs

Taken all together, the addictive drug field and the many new psychoactive drugs are serious challenge for societies and regulations and presently quite difficult to manage.

Cross-References

- ▶ Principles of Analytical Chemistry for Toxicology
- ▶ Prohibition and Restrictions in Regulatory Toxicology
- ▶ Risk Minimization in Drug Development: Regulatory Aspects
- ▶ Specific Toxicity Tests for Neurotoxicity, Immunotoxicity, Allergy, Irritation, Reprotoxicity, and Carcinogenicity

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Recommended Reading

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Tobacco and Alternative Nicotine Products and Their Regulation

82

Manfred Neuberger

Contents

Abstract

Nicotine is highly addictive, interacts with particulates and infection in respiratory disease and promotes cancer in the lung and other organs. Cardiovascular morbidity and mortality were related to fine and ultrafine particles, free radicals, reactive oxygen species, nicotine and other toxins inhaled by smoking, second hand smoking, use of water pipe, heated tobacco, and to vaping e-cigarettes. Steepest increase of risk is observed in the low dose range, explaining the considerable increase of chronic disease by regular passive exposure and its acute hazards for risk groups like persons with ischemic heart disease or asthma. All forms of tobacco are harmful, and there is no safe level of exposure. Oral nicotine does not expose bystanders, but in users of smokeless tobacco cardiovascular disease, oral cancer and pancreatic cancer may increase and like exposure to tobacco smoke in pregnancy it raised rates of stillbirth and low birth weight. Sudden infant death is associated with prenatal and postnatal exposure of the child and also long-lasting effects of parental smoking were observed on

M. Neuberger (✉)
Center of Public Health, Medical University of Vienna, Vienna, Austria
e-mail: manfred.neuberger@meduniwien.ac.at

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respiratory health of children and on the development of their brains. Regulation of all nicotine products should be based on the World Health Organization (WHO) Framework Convention on Tobacco Control. Long-term goal is to raise a nicotine-free generation and to stop selling nicotine products on the free market.

Keywords

Tobacco · E-cigarette · Passive smoking · Nicotine · Health · Disease · Cancer

Introduction

Tobacco kills up to half of its users, more than seven million people worldwide each year as a result of active smoking, and another 1.2 million as a result of involuntary breathing of second-hand smoke (SHS). Compared to earlier reviews (WHO 2008, Fig. 1), annual victims are on the increase and tobacco is projected to kill one billion people this century unless countries take strong action now to prevent it (WHO 2020b).

Tobacco smoke is a complex, dynamic and reactive mixture of hazardous gases and particles, containing thousands of chemicals, including toxic carbon monoxide and highly addictive nicotine. Nicotine promotes cancer growth (Ginzel et al. 2007), initiated by products of pyrolysis (N-nitrosamines, polycyclic aromatic hydrocarbons - PAHs, aromatic amines, volatile hydrocarbons, other organics, heavy metals, and metal compounds). In sidestream smoke (released between puffs from the end of a burning cigarette, cigar, or pipe), particles are smaller and products of pyrolysis higher than in mainstream smoke.

Passive smoking includes inhalation of sidestream smoke and smoke exhaled by smokers. In addition also third-hand smoke deposited on and released from surfaces

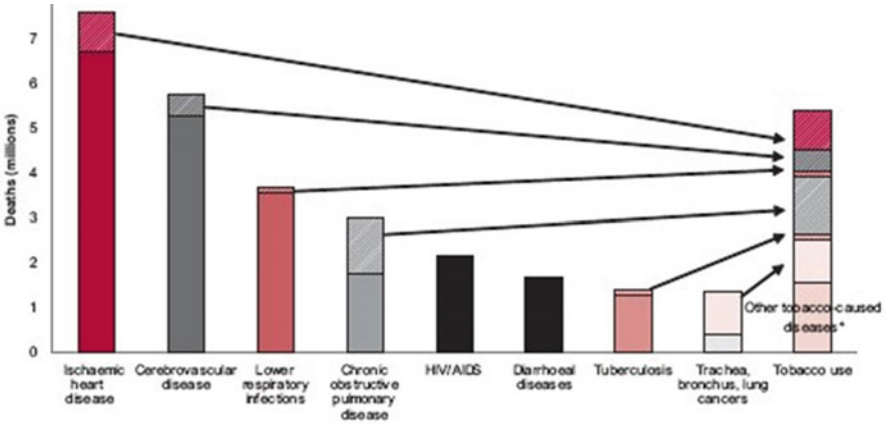

Fig. 1 Eight leading causes of death (2005) and proportions attributable to tobacco smoking © WHO. Reproduced from *WHO REPORT on the global TOBACCO epidemic, 2008* (WHO 2008)

(floor, walls, furniture, house dust) is an involuntary exposure of particular importance for children. Most frequently used are tobacco cigarettes (combustion products), but electronic cigarettes (ecigs) and heated tobacco products (HTPs) are on the increase and many adolescents start their smoking career with these novel products or with water pipe (shisha). Some enter nicotine addiction by oral tobacco like snus[R] or oral nicotine like skruf[R]. All these products are not controlled like nicotine products from pharmacies and all are advertised for continuous use and not for limited treatment. Less products of pyrolysis are emitted by HTPs and ecigs, nevertheless nicotine exposure is hazardous, especially in combination with irritant, toxic, and carcinogenic compounds on the large surface of small particles inhaled by "vapers." Oral and dermal applications of nicotine products do not expose the lungs of users and bystanders with aerosols, but like ecigs oral products can serve as a gateway into nicotine addiction, and some increase of cardiovascular and metabolic disease and cancer (oral cavity, esophagus, pancreas) has been observed after long-term use of oral tobacco. Daily maternal use in pregnancy was associated with increased risk of preterm delivery, stillbirth, neonatal apnea, and higher systolic blood pressure in childhood and altered autonomic cardiac control.

Vascular Effects of Tobacco Products and Nicotine

Most frequent causes of death related to tobacco smoking are from cardiovascular and cerebrovascular disease (CVD). Even SHS triggers acute changes of endothelial function, blood coagulability, heart rhythm, and promotes the development of arteriosclerosis (Neuberger 2019). Main driver of vascular effects seem to be fine and ultrafine particles and toxic compounds.

Both outdoor particle pollution from motor traffic and indoor particle pollution from tobacco smoke trigger endothelial dysfunction, platelet activation, and thrombogenesis (Neuberger 2008). In patients with coronary artery disease, active or passive smoking produces acute myocardial ischemia by adversely affecting the balance of demand for myocardial oxygen and nutrients with myocardial blood supply.

Carbon monoxide (CO) – a major constituent of cigarette smoke – binds avidly to hemoglobin, reducing the amount of hemoglobin available to transport oxygen and impeding release of oxygen by hemoglobin to tissues. CO-reduced exercise tolerance in patients with angina pectoris or intermittent claudication (Neuberger 1979).

Cigarette smoke delivers a high level of oxidizing chemicals to smokers, including oxides of nitrogen and many free radicals and reactive oxygen species (ROS) from both the gas and tar phases of cigarette smoke (Church and Pryor 1985). Exposure to oxidant chemicals in smoke was associated with depletion of endogenous levels of antioxidants like vitamin C (Lykkesfeldt et al. 2000), increased levels of lipid peroxidation products, oxidation of low-density lipoprotein (LDL), inflammation, endothelial dysfunction, and platelet activation (Burke and FitzGerald 2003).

Acrolein, a reactive aldehyde present at high levels in cigarette smoke, forms protein adducts, implicated in loss of protection by high density lipoprotein (HDL)

and in atherogenesis (U.S. CDC 2010). Acrolein also oxidizes antioxidant proteins, which can result in dysfunction and death of endothelial cells, contributing to atherosclerosis. In addition, acrolein inhibits antithrombin activity and could contribute to smoking-induced coronary vasospasm.

Nicotine has a central role in CVD from tobacco use and from SHS. It is a sympathomimetic drug that increases heart rate and cardiac contractility, transiently increasing blood pressure and constricting coronary arteries, which is most risky in persons with preexisting hypertension. Nicotine also contributes to endothelial dysfunction, insulin resistance, and lipid abnormalities. Both tobacco use and SHS are risk factors for diabetes and aggravate insulin resistance in persons with diabetes. The mechanism appears to involve the effects of oxidizing chemicals in the smoke and the sympathomimetic effects of nicotine. Combined effects of smoking and ambient air pollution on CVD are more than additive (Fig. 2): From the hazard ratios for CVD deaths the dominant influence of smoking can be seen, but also additional increases from ambient air pollution with PM2.5, which are higher in current smokers than in never-smokers.

Combined toxins distributed on the large surface of (ultra)fine smoke particles trigger pulmonary reflexes and inflammation, reach the alveoli, some of them cross the alveolar-capillary membrane, elicit acute endothelial dysfunction with inactivation of nitric oxide (mediating vasodilatation), impair the viability of endothelial cells and reduce the number and functional activity of circulating endothelial

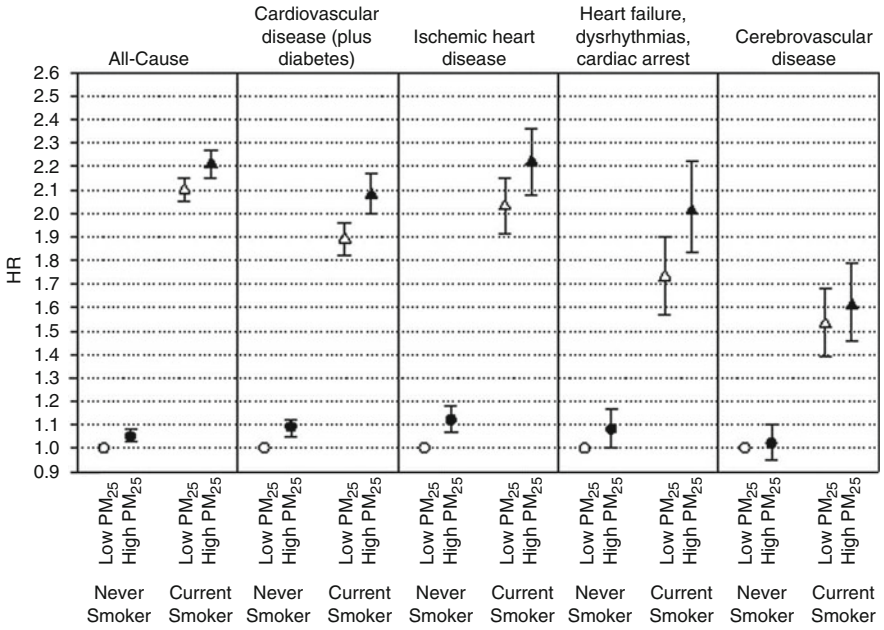

Fig. 2 Cardiovascular mortality: Hazard ratios for active smoking and for ambient air pollution with fine particles – PM2.5 (Turner et al. 2017). ©Elsevier, with kind permission

progenitor cells. Platelets of non-smokers appear to be susceptible to quick pro-aggregatory changes with every SHS exposure. Apart from vasoconstriction and thrombus formation from sticky platelets, increased fibrinogen and other factors of blood coagulation, further impairment of the myocardial oxygen balance is promoted by nicotine-induced adrenergic stimulation and particle-induced autonomic dysfunction with heart rhythm disturbances and worsening of left ventricular diastolic function. Experiments in healthy men showed that a 30-min exposure to SHS (e.g., the time of a meal in a smoking room) was sufficient to reduce coronary flow velocity reserve (Otsuka et al. 2001) and sustained vascular injury characterized by mobilization of dysfunctional endothelial progenitor cells with blocked nitric oxide production and activation of platelets in blood. Chronic vascular effects of SHS start with endothelial dysfunction in children, arterial stiffness, and the development of a thickening of the intima-media and other signs of early atherosclerosis. Combined effects of tobacco smoke with ambient air pollution with urban aerosols have been detected and complex interactions with nutrition are likely, especially in connection with diabetes. SHS is a risk factor for metabolic syndrome, glucose intolerance, insulin resistance, and the development of type 2 diabetes mellitus. A meta-analysis by Wei et al. 2015 on seven prospective studies investigating the risk for developing type 2 diabetes from SHS found a RR of 1.33 (95% CI 1.20–1.46) and after adjustments for publication bias 1.27 (95%CI 1.16–1.40). After manifestation of diabetes vascular complications are increased by further exposure to tobacco smoke.

Accelerated atherosclerosis from tobacco smoke may result in ischemic heart disease, increased risk of acute myocardial infarction, and sudden cardiac death, stroke, peripheral artery disease with gangrene of extremities, aortic aneurysm, atrial fibrillation, pulmonary embolism, and other sequelae (Banks et al. 2019). The largest smoking-related disease burdens in adults are from CVD, with highest RR at young ages, but continuous increase of risk with pack-years, age, and years of smoking. Over the years also regular passive smokers build up a considerable risk for ischemic heart disease. Classifying male non-smokers in the lowest quarter of serum cotinine (0–0.7 ng/ml) as "light passive smokers" and non-smokers within the upper three quarters of cotinine concentrations (0.8–14 ng/ml) as "heavy passive smokers," Whincup et al. (2004) observed that the risk for major coronary heart disease increased by years of follow-up significantly steeper in heavy passive smokers, comparable to the increase in light active smokers, consuming 1–9 cigarettes per day.

In a European cohort on circulatory mortality a hazard ratio of 1.25 (95%CI 1.04–1.50) was calculated for passive smoking (verified in a subsample by plasma cotinine) per each additional daily hour of exposure (Gallo et al. 2010). A meta-analysis found for SHS a relative risk of 1.35 (95% CI: 1.22–1.50) for stroke and 1.27 (95% CI: 1.10–1.48) for ischemic heart disease, with higher risks in women (Fischer and Kraemer 2015). Many studies underestimated relative risks for CVD, because they compared active smokers with non-smokers, of which many were exposed to SHS. If choosing non-smokers without exposure to SHS as the proper control group (Bonita et al. 1999), the odds ratio of stroke from active smoking increased from 4.14 (95% CI 3.04–5.63) to 6.33 (95% CI 4.50–8.91). Flores et al. (2016) found that the premature mortality hazards of recalled and unconscious

exposure to SHS are comparable and predicted by serum cotinine at beginning of observation. There was a significant increase in years of life lost, adjusted for confounders, across cotinine categories, similar in non-smokers reporting SHS and in non-smokers reporting no SHS exposure.

Dose-response relationships are not linear, but a much steeper increase of cardiovascular risk is observed in the low-dose range, covering SHS and occasional active smoking. SHS increases the risk of coronary heart disease by about 30%. In occasional smokers consuming an average of less than one cigarette per day a hazard ratio of 1.71 (95% CI 1.33–2.21) was found for CVD (Inoue-Choi et al. 2017) and the consumption of 1–4 cigarettes per day raised relative risk of dying from ischemic heart disease to 2.74 (95% CI 2.07–3.61) in men and 2.94 (95% CI 1.75–4.95) in women (Bjartveit and Tverdal 2005). Disproportionately, high risk at low levels of exposure suggest that there is no safe lower limit of SHS exposure for risk groups. Even if reduction of active smoking is sustained, the reduction of daily cigarettes smoked reduces the risk only marginally compared to complete cessation.

Most at risk for acute effects of SHS are patients with preexisting coronary or cerebrovascular diseases, which in turn are promoted by chronic exposure to SHS. Since Sargent et al. (2004) reported reduced incidence of admissions for myocardial infarction after a public smoking ban, numerous studies confirmed that enforcement of smoke-free laws rapidly reduces admissions for acute coronary syndrome and other cardiac and cerebrovascular diseases (Neuberger 2008). Smoke-free legislation is associated with a lower risk of hospitalization and death from CVD, significantly lower rates of hospital admissions or deaths from coronary events (relative risk, 0.85; 95%CI 0.82–0.88), other heart disease (relative risk, 0.61; 95%CI 0.44–0.85), and cerebrovascular accidents (relative risk, 0.84; 95%CI 0.75–0.94). More comprehensive laws were associated with larger changes in risk. Indoor smoking bans reduced myocardial infarction by 10–20%, in the first year mainly associated with the elimination of passive smoking and followed by sustainable decreases of coronary syndrome, myocardial infarction, stroke, and incident diabetes also in ex-smokers (Tan and Glantz 2012; Akter et al. 2015).

Shisha (water pipe, hookah, narghile) produces similar risks for users and bystanders as tobacco cigarettes, but concentrations of carbon monoxide in charcoal-heated shisha and of heavy metals are higher. Depending on intensity and duration of exposure, acute CO intoxications were reported and in long-term similar CVDs can develop as in cigarette smoking (Waziry et al. 2017). Animal experiments showed hypercoagulability, inflammation, as well as systemic and cardiac oxidative stress (Nemmar et al. 2017). Also the smoking of other pipes and cigars, whether active or passive, carries significant risk of smoking-related CVD (Shaper et al. 2003).

Because less products of pyrolysis are formed by heating than by burning tobacco, HTPs are marketed as less dangerous than conventional cigarettes; however, biomarkers of potential cardiovascular harm did not support this claim (Glantz 2018). HTPs impair vascular endothelial function measured by arterial flow-mediated dilatation in rats to the same extent as by cigarette smoke (Nabavizadeh et al. 2018). An important advantage of all electronic devices over conventional cigarettes

is that SHS or second-hand aerosol is only produced when the user exhales and not continuously like in conventional smoking between puffs. The doses calculated for second-hand uptake of particles, toxins, and carcinogens from electronic devices are usually much lower than from traditional tobacco products, below 1.6×10^8 particles/kg bodyweight, but dosimetry estimates were 50–110% higher for HTPs than for ecigs (Protano et al. 2017). The carrier function of aerosols from electronic devices might be similar, but the clearance of liquid particles is certainly faster than of solid, carbonaceous particles released by conventional cigarettes. On the other hand, some electronic devices release even more ultrafine aerosols carrying tobacco-specific nitrosamines and heavy metals and also volatile organic compounds are released, which are harmful for users and bystanders. In addition hemodynamic changes and adverse effects of nicotine on blood lipids, as well as the induction of insulin resistance contribute to cardiovascular risk of HTPs and ecigs (Zhang et al. 2018; Protano et al. 2020).

Electronic cigarettes (ecigs) have been called "a wolf in sheep's clothing," because they may serve as a gateway drug for youth, prolong nicotine addiction, and the ritual in smokers who would otherwise be willing to quit. So they keep up the handling and use of cigarettes in public and the denormalization of cigarettes is undermined (Neuberger 2015). Even bystanders absorb considerable amounts of nicotine (Ballbè et al. 2014) and inhale particles, which are smaller than in SHS (Schripp et al. 2013). Aerosols exhaled during vaping are less persistent than SHS, nevertheless they are carriers for toxins, which they adsorb on their large surface and transport them to persons in the same room and even to neighboring rooms (Khachatoorian et al. 2018), and into the depth of the lung, where clearance is less efficient and where ultrafine particles can reach the bloodstream. Most dangerous is the contamination of small rooms like passenger cars (Schober et al. 2019). WHO (2020a) clearly stated that ecigs (electronic nicotine delivery systems – ENDS and electronic non-nicotine delivery systems – ENNDS) and other vaping products are dangerous for both users and bystanders exposed to the aerosol. Cohort studies on long-term effects are still missing, but similar cardiovascular risks seem to exist as from tobacco products and HTPs, indicated by a sustained hyperadrenergic effect (triggering arrhythmias and other harmful cardiac reactions), oxidative stress and activation of inflammatory pathways (leading to atherosclerosis, plaque progression and instability, and myocardial ischemia), endothelial dysfunction (leading to impaired nitric oxide release and oxygen supply to tissues), arterial stiffness (caused by nicotine and predicting CVD), and pathological thrombus formation from platelet activation (Middlekauff 2020). The chronic use of ENDS produced platelet aggregation to a similar degree as in chronic users of tobacco products (Nocella et al. 2018). Most worrying are first cross-sectional studies showing associations of ecigs use with myocardial infarction (Alzahrani et al. 2019). Many users of ecigs return to tobacco cigarettes or become dual users and dual use was associated with higher odds of CVD than smoking tobacco only (Osei et al. 2019). Cohort studies on long term risks of use and second hand inhalation of ecigs and HTPs are still lacking, but from the indicators mentioned above it has to be assumed that also passive vaping increases CVD. Because of risks for CVD, respiratory disease, cancer, and addiction

(see below), HTPs and ecigs cannot be recommended as a help for smoking cessation, even though health risks of smoking and SHS are even higher. Some ecigs are labelled "without nicotine," despite of releasing considerable amounts. Adjustable voltage, nicotine salts, and additives were developed to increase nicotine uptake and to mimic the rapid transfer of nicotine to blood and brain like in smoking. For smoking cessation, dermal and oral nicotine products from pharmacies are safer for nicotine replacement and do not contaminate the breathing air of bystanders. Their nicotine content is controlled and can be reduced gradually during cessation. In young and light smokers, preference should be given to cessation aid without any nicotine. Also for pregnant women any form of nicotine is harmful for mother and child (Ginzel et al. 2007). Higher systolic blood pressure in childhood and altered autonomic cardiac control were associated with nicotine consumed by the mother in pregnancy, whether by inhalation or by oral products (Watanabe and Parikh 2019). Stop of oral tobacco (snus) use after a myocardial infarction nearly halved mortality risk, similar to the benefit associated with smoking cessation (Arefalk et al. 2014).

Respiratory Effects of Tobacco Products and Nicotine

Tobacco smoking is the leading preventable cause of death and disability, with important contributions of respiratory diseases (Fig. 1). The earlier smoking is stopped, the better are respiratory outcomes. To end accelerated decline of lung function compared to non-smokers, smokers need complete smoking cessation. Similar to CVD, reduction of daily consumption to below 5 cigarettes is insufficient to stop respiratory function decline (Fig. 3).

In industrialized countries smoking prevalence decreased, nevertheless some studies attributed up to two-thirds of deaths in current smokers to tobacco and an even higher number of diseases, which are not registered as main cause of death (Banks et al. 2019). In respiratory diseases, asthma and chronic obstructive pulmonary disease (COPD) from smoking are most important for disability, early retirement, and invalidity. In 2015, COPD caused 2.6% of global disability adjusted life years (DALYs) and asthma 1.1% of global DALYs, with smoking, air pollution, and SHS as the main risk factors (Soriano et al. 2017). Smokers had a fourfold increase in risk of developing COPD (Bellou et al. 2019), and there are combined effects of active and passive smoking with other environmental and occupational air pollution.

Other important interactions of tobacco smoke in respiratory diseases occur with infections, especially in childhood. Smokers are touching their face and mouth more often and they can also transfer bacteria causing meningitis or viruses causing respiratory disease by kissing a child. Combined effects of smoking and tuberculosis are a world-wide problem (Fig. 1), especially in the developing world. Smoking and SHS impair mucociliary clearance and lung function. By reducing defense of the respiratory system, weakening and damaging it, tobacco smoke enables viruses and bacteria to invade. Some infectious diseases become more serious or even lethal, e.g., pneumonias in children from SHS or bronchiolitis from respiratory syncytial virus. Higher mortality was also reported in smokers from influenza and corona virus

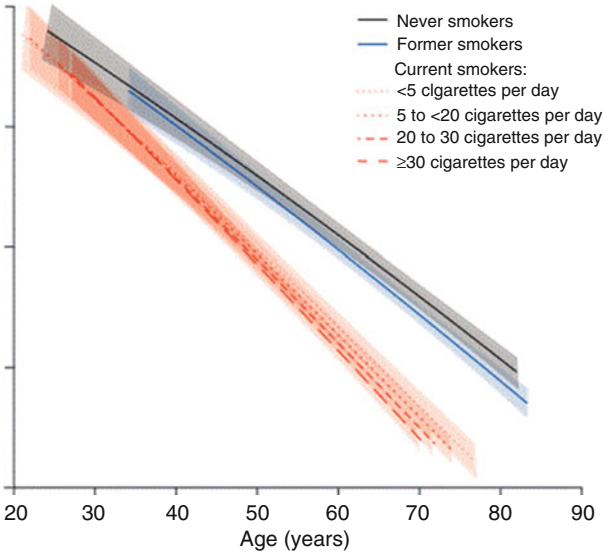

Fig. 3 Decline of Forced Expiratory Volume in first second (FEV1) by age and smoking (Oelsner et al. 2020). ©Elsevier with kind permission

infection (Middle East Respiratory Syndrome and COVID-19), especially in smokers with COPD (Alqahtani et al. 2020).

Also HTPs and ecigs aerosols contain numerous respiratory irritants and toxicants, triggering increased airway hyperreactivity, distal airspace enlargement, mucin production, as well as cytokine and protease expression. Just 15 min of exposure to ecig aerosol induced transient lung inflammation and impaired gas exchange in healthy volunteers. Defense by ciliary function, neutrophils, and alveolar macrophages is impaired and cross-sectional studies found an increase of asthma and of bronchitis symptoms, even in adolescents who had never used tobacco. Long-term observations are still missing, but experiments with animals and with human tissues showed similar structural lung changes from ecigs as in the development of COPD from tobacco, protease release from alveolar macrophages and blood neutrophils, and nicotine dependent elastase release (Gotts et al. 2019). Respiratory toxicity of ecigs is determined by a large number of aromas, nicotine, and the carriers propylene glycol, vegetable glycerin, and other solvents forming aerosols with small particle diameter. Heavy metals (Ni, Cr, etc.) contribute to respiratory risks of ecigs (Fowles et al. 2020). Some ingredients, additives or their decomposition products might cause special respiratory diseases, e.g., diacetyl, which caused bronchiolitis and "popcorn lung" in occupational exposures. In 2019 in the USA, 2558 persons were hospitalized and 60 patients died from acute vaping disease, called "e-cigarette, or vaping, product use–associated lung injury" (EVALI), characterized by gastrointestinal and severe respiratory symptoms with bilateral pneumonia and Acute Respiratory Distress Syndrome (Werner et al. 2020). Days

to weeks after consumption of ecigs also, young people without previous lung disease and without detectable infection developed respiratory failure with pulmonary edema, hypoxemia, diffuse alveolar & endothelial damage, protein-rich inflammatory fluid in alveolar space, and need for mechanical ventilation. The heterogeneity of clinical appearance might resemble the high variety of uncontrolled additives used in ecigs. Most (but not all) cases of EVALI had used ecigs with cannabis or tetrahydrocannabinol (THC). These mixtures contained vitamin E acetate, used as a thickening agent in THC products, which was also found in bronchoalveolar lavage of patients. At temperatures reached in vaping devices the exceptionally toxic ethenone (C_2H_2O) and other ketenes may be formed by pyrolysis of vitamin E acetate (Wu and O'Shea 2020), possibly amplified by metal catalysts. Since the outbreak of SARS-CoV-2 epidemy new cases of EVALI are easily misdiagnosed as COVID-19 (Armatas et al. 2020).

Cancer from Tobacco Products and Nicotine

Tobacco smoke contains dozens of carcinogens, including class 1 carcinogens like benzo[a]pyrene, N′-nitrosonornicotine (NNN), nicotine-derived nitrosamine ketone (NNK), 2-toluidine, 2-naphthylamine, 4-aminobiphenyl, benzene, 1,3-butadiene, ethylene oxide, vinyl chloride, and metals like As, Be, Cd, Cr(VI), and ^{210}Po. Also other products of tobacco pyrolysis contribute to cancer risks in many organs, e.g., benz[a]anthracene, dibenz[a,h]anthracene, formaldehyde, N-nitrosodimethylamine, N-nitrosodiethylamine, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), acrylamide, glycidol. From these genotoxic compounds or their metabolites cancer can develop in lungs, bronchi, trachea, larynx, mouth, throat, esophagus, stomach, colon, rectum, liver, pancreas, kidney, renal pelvis, bladder, uterine cervix, or blood (acute myeloid leukemia). Combined effects of tobacco smoke with alcohol are of importance in cancer of the upper digestive tract, larynx, colon, liver, pancreas, and female breast, and with chronic infection, e.g., with human papilloma virus (HPV) in cervical cancer and with hepatitis B or C in hepatocellular carcinoma. Increased risk of tobacco smoking was also found for mucinous ovarian cancer, chronic myeloid leukemia, and for breast cancer after exposure (active or passive) during the time when the female breast develops. Frequent and intensive SHS is a verified risk for cancer of the lung and of other locations (breast, pancreas, bladder, etc.), subject to similar combined effects as active smoking.

Smoking of pipes and cigars was found associated with cancer of lung, larynx, oral cavity, hypopharynx, esophagus, pancreas, stomach, and urinary bladder. Waterpipe does not filter carcinogens and the nicotine load of one pipe corresponds to the inhalation of approximately 10 cigarettes. Additional carcinogens enter the smoke of shisha from the burning of coal. Some PAHs, benzene, and heavy metals like Cd, Cr^{VI}, Ni, Co, As are higher in shisha smoke than in cigarette smoke and put users and persons sharing the same room at risk for cancer. In users metabolites of tobacco-specific nitrosamines were detected in urine and in regular shisha users

studies found an increase of cancers of the head and neck, esophagus, and lung (Mamtani et al. 2016).

Cigarette smoking is the number one cancer risk, largely due to lung cancer (Shaper et al. 2003). Cigarette smokers are 15–30 times more likely to get lung cancer or die from lung cancer than people who do not smoke. Even smoking a few cigarettes a day or smoking occasionally increases the risk of lung cancer (Bjartveit and Tverdal 2005). Lung cancer increases with packyears and decreases after smoking cessation much more slowly than the cardiovascular risk. Smoking attributable lung cancer amounts to 70–90%. Other causes are particulate air pollution, other environmental exposures indoors (residential radon, SHS), and occupational exposures. Some combined effects of smoking are overadditive (radon, amphibole asbestos), so that attributable cancer risks sum up to more than 100% and high decreases of lung cancer risk can be achieved by elimination of only one risk factor.

As a defense against microbes, macrophages react to small particles with the induction of nitric oxide and reactive oxygen intermediates. Like in diesel soot the carcinogenicity of tobacco smoke seems to be less dependent on the mass of carcinogens than on the surface of small particles by which traces of genotoxic substances are transported into the lung. The particle size of SHS is smaller and on its large surface products of pyrolysis are transported, which are formed in higher concentrations in sidestream smoke between puffs than in mainstream smoke. Important for cancer development is also nicotine, which is not only genotoxic on human epithelia of the upper aerodigestive tract (Sassen et al. 2005), mediated through oxidative stress (Bavarva et al. 2004), but is also an important epigenetic risk, promoting cancer cell proliferation by several mechanisms, inhibiting apoptosis and thereby increasing cancer cell survival, stimulating angiogenesis, and thereby vascularization of the growing tumor, and promoting cancer migration (Fig. 4). Possible promotion of a smoking induced cancer by nicotine is one of the reasons, why nicotine replacement therapy after smoking cessation should be limited in time (Ginzel et al. 2007). The potent lung carcinogens NNK and NNN can form during blending of tobacco, but also in the smoker by endogenous nitrosation. Smoking itself increases nitrogen oxides and other nitrosating agents in saliva, stomach, urine, etc. For endogenous nitrosation of nicotine to NNN and NNK, the amount of ingested nitrite and nitrate is less important than chronic infection and diseases with chronic inflammation, which cause continuous increase of NO and peroxynitrite, capable of forming nitrosamines from nicotine and aminoketone (Parzefall et al. 2005).

Cancer risks of HTPs and e-cigarettes are lower than from tobacco products, but not negligible. Certainly the mass of carcinogens in aerosols of HTPs and ecigs is much smaller than in tobacco smoke, but traces of genotoxic agents might be sufficient to induce cancer if distributed on the large surface of very small particles in these aerosols and combined with potent promoters like nicotine (see above). Propylene glycol, glycerol, and flavors in ecigs produce free radicals and reactive oxygen species (Bitzer et al. 2017). Depending on voltage and temperature propylene glycol produces formaldehyde and its hemiacetal in the aerosol (Jensen et al. 2015). Ni, Cr, and other heavy metals were found to be transferred from the heating coils to the e-liquids (Fowles et al. 2020) and some of these carcinogenic metals

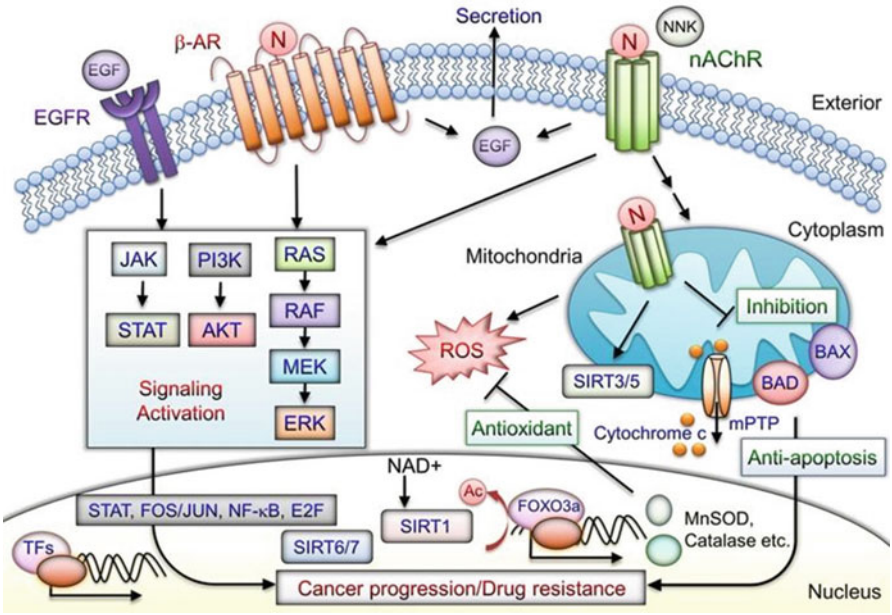

Fig. 4 Lung cancer promotion by nicotine, signaling pathways downstream of nAChRs promoting drug resistance and antiapoptosis (for abbreviations see Cheng et al. 2020). Creative Commons License. Cheng et al. 2020

(from cartridge, fluid, etc.) were higher in e-cigarette aerosols than in SHS (Zhao et al. 2020). N-nitrosamines like NNN and their metabolites were found in saliva and urine of vapers (Bustamante et al. 2018). Also some of the sugars and aromas added to attract children can decompose to carcinogens. Bavarva et al. (2004) found nicotine induced mutations in human breast cells and other epithelial cells across 1,585 genes, of which 49% were associated with cancer, with genes of the mucin family among the top mutated genes. 26% of genes linked to cancer development are deregulated both in buccal mucosa of vapers and of smokers, partly different genes, which are implicated in cancer of lung, esophagus, bladder, ovary, and leukemia (Tommasi et al. 2019). Martin et al. (2016) found decreased expression of immune-related genes in the nasal mucosa of vapers, 53 genes like in smokers (some more suppressed), and 305 genes in vapers only. Damaged DNA and impaired DNA repair from exposure to e-cigarette aerosol was found in mice (lung, heart, bladder) and in human lung cells and bladder cells (Lee et al. 2018).

Oral uses of smokeless tobacco have the advantage that there is no inhalation risk for users and bystanders, but carcinogens like tobacco-specific nitrosamines are a cancer risk for betel quid chewers and for users of oral tobacco. Overall, there is sufficient evidence that smokeless tobacco causes oral cancer and pancreatic cancer in animal studies and in humans (Cogliano et al. 2004), possibly also esophageal cancer. Levels of cotinine and metabolites of tobacco-specific nitrosamines in urine

were found higher in smokeless tobacco users than in smokers (Hecht et al. 2007). Most risky is the combination of oral tobacco with tobacco smoking.

All nicotine products promote carcinogenesis and may interfere with cancer therapy. By example human pharyngeal cancer cells became resistant to Cisplatin by exposure to ecigs aerosol (Manyanga et al. 2019). Figure 4 shows some of the pathways by which nicotine, the primary addictive constituent of cigarettes, contributes to cancer progression through activation of nicotinic acetylcholine receptors (nAChRs), which are membrane ligand-gated ion channels. Activation of nicotine/nAChR signaling is associated with lung cancer risk and drug resistance (Cheng et al. 2020).

Other Important Diseases Promoted by Tobacco and Nicotine Products

Numerous other diseases leading to disability were associated with smoking tobacco and with exposure to SHS. For newly developed nicotine products, there is still a lack of epidemiological data on their effects on chronic diseases, but from animal experiments and bioindicators in humans, there is sufficient evidence for metabolic effects of tobacco smoke and of nicotine. HTPs showed no difference in most biomarkers of potential harm from conventional cigarettes (Glantz 2018), possible hepatotoxicity and a potential for unexpected organ toxicity not previously associated with cigarettes (Chun et al. 2018).

Smoking is linked to the development of rheumatoid arthritis and their severity and exacerbation. Animal experiments proved that nicotine causes specific alterations in the disease-related cellular and humoral immune responses and exacerbate inflammatory arthritis (Lee et al. 2017). Smoking has also been identified as a major risk factor for osteoporosis, resulting bone fractures and impaired healing. Nicotine affects estrogen and calcium metabolism, osteocyte development and bone lamellar structure, inhibits catalase and glutathione reductase activity, contributing to an accumulation of ROS by cigarette smoke exposure, and induces apoptosis in human osteoblasts (Marinucci et al. 2018).

Smoking or heavy exposure to SHS increase the risk of blindness, macular degeneration, and a number of other eye diseases (dry eye, cataracts, uveitis, diabetic retinopathy, glaucoma with optic nerve damage). For some of these diseases, impairment of microcirculation by nicotine plays an important role, which can be triggered also in healthy subjects by oral nicotine (Cınar et al. 2019).

Effects of Tobacco Products and Nicotine on Fetus and Child

Maternal smoking during pregnancy is associated with numerous adverse offspring outcomes (Horak et al. 2012). Complications of gravity, birth, and reduced birth weight are mainly seen if pregnant smokers continue to smoke into the last trimester,

but the problem of tobacco smoke exposure starts with conception since active smoking reduces the sperm count, male and female fertility. Impairments of fetal life cannot only be measured by higher rates of abortion and stillbirths, but also by increased fetal heart rate, limb movements, and fetal activity associated with later infants' attention and self-regulation (Stroud et al. 2019). Neonatal morbidity and mortality and malformations (orofacial clefts) are increased, and birthweight is decreased by an average of 200 g. The leading cause of death in the first year is sudden infant death syndrome (SIDS), which doubles when the mother smokes one cigarette per day and increases ninefold if she smokes more than 20 cigarettes per day. Also long-term neurobehavioral deficits develop more frequently, in particular attention deficit hyperactivity disorder, but also addictive behavior, and disruptive behaviors/conduct disorder. Also lung development of children is impaired by maternal smoking during pregnancy and afterwards by parental smoking (Pattenden et al. 2006) and the risk for infectious and allergic respiratory disease and asthma increases. A meta-analysis on parental smoking and the risk of congenital heart defects concluded, that maternal active smoking was significantly associated with risk of atrial septal defect and right ventricular outflow tract obstruction and that also maternal passive smoking as well as paternal smoking increased the risk of congenital heart defects in offspring (Zhao et al. 2019). Many effects of prenatal exposure to tobacco smoke have been attributed to nicotine (Ginzel et al. 2007), with adverse perinatal outcomes associated to placental syndromes and direct toxic effects on arteries supplying the fetus and his heart. Prenatal exposure to constituents of tobacco smoke can also have long lasting effects on children like metabolic disease and visceral adiposity, but only few epidemiological studies were able to disentangle them from effects of postnatal exposure. Transgenerational effects of smoking in pregnancy seem to be related to altered DNA methylation patterns in cell nucleus and mitochondria, which are gender-specific and tissue dependent. Epigenetic impacts of in utero exposure to maternal smoke, e-cigarette aerosol, and maternal SHS on fetal respiratory development may persist across subsequent generations, regardless of the smoking habits of the second generation, so that asthma in grandchildren was associated with the smoking habit of the grandmother in pregnancy (Zakarya et al. 2019).

Also passive smoking of the mother puts the fetus at risk of growth retardation and shortening of gestational age. Individual risks for mother and child from mothers' passive smoking are substantially lower than from her active smoking. But since the number of exposed pregnancies are higher, the number of attributable cases is in the same order of magnitude as for active smoking in pregnancy. Postnatal exposure of children to parental smoking was estimated to cause approximately 10% of children's respiratory and middle ear diseases (Moshammer et al. 2007).

SHS leaves accumulating contaminants on surfaces like carpets, wallpapers, upholstery, blankets or soft toys and these remnants called "Third Hand Smoke (THS, Cold Smoke)" endanger in particular children by oral, dermal, and inhalation uptake from house dust, etc. (Neuberger 2018). Even parents omitting contamination of indoor air nevertheless bring toxins and carcinogens to indoor

spaces and to their children by clothes, hair, skin, and breath, but the highest contamination is found on surfaces of rooms used for smoking. From these surfaces toxins are released back into the air and by aging and chemical transformations more toxic pollutants are formed, e.g., residual nicotine from tobacco smoke adsorbed to indoor surfaces reacts with ambient nitrous acid to form carcinogenic nitrosamines. Animal experiments demonstrated numerous effects of THS: hyperactivity, persistent changes in the immune and hematopoietic system, lung cancer, liver damage, increased thrombogenesis, and metabolic effects, including elevated triglycerides, increased LDL, decreased HDL, and insulin resistance through oxidative stress. Estimates of harm from THS are all by inference, since direct evidence of human health problems arising from THS is still missing (Neuberger 2019).

Tobacco cigarettes carry the highest risk for parents and child, but any use of nicotine in pregnancy should be discouraged, because for many of the fetal hazards described above (SIDS, brain development, lung development, etc.) nicotine plays an essential role (Ginzel et al. 2007). Rodent and primate studies found that the effects of maternal SHS exposure on developmental outcomes are comparable to effects seen with isolated nicotine, including effects on number of neurons, neuronal and synaptic damage, and cognitive dysfunction. The motivation to stop smoking during pregnancy should be supported, but nicotine replacement was unsuccessful and has adverse effects on fetal development. Even more dangerous are ecigs without proper control of nicotine dose and problematic additives. Maternal smokeless tobacco use increased rates of stillbirth, low birth weight, and altered the male/female live birth ratio (Ratsch and Bogossian 2014).

Nicotine exposure during adolescence altered development of cerebral cortex and hippocampus and was associated with deficits in working memory, attention, and auditory processing, as well as increased impulsivity and anxiety (England et al. 2017). First nicotine exposure of children is usually by SHS, which could lead to later active smoking not only by imitation of parent's behavior and easy access of cigarettes, but also by providing a molecular basis for later nicotine addiction and possibly other addictions (Kandel and Kandel 2014).

Addiction

Nicotine is a highly addictive drug, by smoking rapidly absorbed into the blood and delivered quickly to the brain, so that nicotine levels peak within 10 s of inhalation, releasing the neurotransmitter dopamine in the brain and activating reward pathways which regulate reinforcement and feelings of pleasure. This happens with every puff in cigarette smoking, but the smoker adapts by increasing nAChRs and as a result needs to increase the number of cigarettes and the depth and duration of inhalation in order to sustain slight, brief euphoria. Even after this euphoria had disappeared completely over the years, the smoker still longs for the next cigarette, which quickly relieves withdrawal symptoms like irritability, craving, depression, anxiety, cognitive and attention deficits, sleep disturbances, and increased appetite. These

withdrawal symptoms may begin within a few hours after the last cigarette, quickly driving people back to tobacco use, which has also been learned meanwhile to be used as a coping strategy for stress and – like in classical conditioning – prompts associations with all kinds of environmental stimuli and things we desire.

Given the rapid rise of nicotine and associated psychoactive effects, smoking of combustible cigarettes allows the smoker to titrate the level of nicotine and related effects during smoking. This makes smoking the most reinforcing and dependence-producing form of nicotine administration. Nicotine products absorbed more slowly are less addictive, but the tobacco industry attempts to achieve a quick rise in brain nicotine concentrations also by other products, e.g., nicotine salts with organic acids like benzoic acid, which make ecigs taste milder, so that users can inhale deeply and reach nicotine levels in blood and brain within 5 min, which are comparable to combustion products. This way ecigs can become a gateway drug into nicotine addiction for young non-smokers, and for smokers ecigs make it more difficult to leave nicotine addiction, frequently resulting in dual use of ecigs and tobacco cigarettes. Replaceable podstyle nicotine cartridges have also become popular ecigs among youth because of efficient nicotine delivery, appealing flavors, sleek designs, ease of concealment, and social media marketing (Lee et al. 2020).

In smokeless tobacco products nicotine is absorbed through mucous membranes in the mouth and reaches peak blood and brain levels more slowly than from inhalation, why they are less addictive. Nevertheless also smokeless tobacco products proved to cause addiction and are most dangerous if combined with tobacco cigarettes. Products for skin application are absorbed much slower, are much less addictive, and should therefore be preferred, if nicotine replacement is needed at all. Only for heavy smokers, fast absorbed nicotine products like a gum are necessary, usually in combination with a nicotine patch, but for a limited time only. Help in smoking cessation is possible without nicotine (varenicline, bupropion, cytisine, etc.) and all pharmaceutical help must be accompanied by professional counseling, because motivation is the most important part in the treatment of smokers.

Though nicotine exerts a priming effect on illegal drug use through global acetylation in the striatum, creating an environment primed for the induction of gene expression (Kandel and Kandel 2014), only a small part of nicotine addicts turn to illegal drugs; however, most persons addicted to marihuana (cannabis), cocaine and similar drugs are also addicted to nicotine and the majority started with nicotine in their career of addictions. These addicts also continue to use nicotine, which may enhance the physiological effects of other drugs.

From animal research, a number of additives in combustible cigarettes and ecigs are known to increase the reinforcing properties of nicotine, e.g., acetaldehyde created by the burning of sugars (added as sweeteners for children) or monoamine oxidase inhibitors (Hogg 2016). Cooling of the smoke like in shisha or adding of aromas like menthol makes deep inhalation easier, resulting in higher nicotine concentration in blood and brain and speeding the transition from occasional to regular smokers.

Guidelines and Regulation on Tobacco Products and Nicotine

The most important guidelines are connected to the WHO Framework Convention of Tobacco Control (FCTC), an evidence-based global treaty signed and ratified by 181 nations and the European Union. This convention was adopted during the 56th World Health Assembly in 2003, entered into force in 2005 and is supplemented by details at regular meetings of the meanwhile 182 parties. A key element is article 5.3, which states that there is a fundamental and irreconcilable conflict between the tobacco industry's commercial interests and public health policy interests. FCTC is raising awareness of international instruments that governments should use to protect tobacco control policies from tobacco industry interference. Politicians and officials need to limit interactions, communications, and contacts with the tobacco industry to those necessary for effective regulation and to make them transparent for civil society. This is an essential prerequisite for reduction of the demand for tobacco (Art. 6, 7), for protection from SHS (Art. 8), for regulation of constituents and emissions of tobacco products (Art. 9) and their packaging and labelling (Art. 11), as attempted in the EU Tobacco Products Directive (TPD) 2014/40/EU. The TPD was a progress for public health limiting the sale and merchandizing of tobacco and related products in the EU, but was weakened because FCTC Art. 5.3 had not been implemented before in member states. Also a comprehensive ban of all tobacco advertising, promotion, and sponsorship (Art. 14) is still missing in many countries and very limited budgets are available for promotion of non-smoking by media campaigns and educational programs (Art. 13), compared to the huge amounts of money the international tobacco industry is investing in direct and indirect tobacco advertising. FCTC also recommends a number of measures to reduce the supply of tobacco, e.g., elimination of all forms of illicit trade in tobacco products (Art. 15). An own protocol on illicit trade tracking, tracing, etc. entered into force in 2018, was ratified by 61 nations meanwhile and details are updated regularly. Youth protection from commercial interests of the tobacco industry and retailers (Art. 16) is still insufficient in many countries and several surveys showed that the age control by tobacconists is insufficient and the electronic age control by vending machines is unreliable (Berger and Neuberger 2020). Unacceptable are also the serious risks posed by tobacco growing to human health and to the environment (Art. 17). Child labor in tobacco growing is aggravated by nicotine poisoning, because during harvesting nicotine of the wet, green leaves gets absorbed through the skin of the children. Common agricultural practices in tobacco farming, especially in low- and middle-income countries, lead to deforestation and soil degradation, agrochemical pollution, destruction of ground water resources, sedimentation of rivers, reservoirs, and irrigation systems, which in turn lead to ecological disruptions that cause a loss of ecosystem services, including land resources, biodiversity, and food sources, all of which negatively impact human health (WHO 2017). All phases of cigarette production, from leaf cultivation through cigarette manufacture to transportation, contribute to environmental hazards and greenhouse gas emission responsible for global climate change. Cigarette butts are toxic waste, endangering playing children, polluting water and air, and finally ending as microplastics in the ocean. E-cigarettes

and their batteries are a new and growing environmental threat, which would need regulation.

Besides FCTC and policy options convened at the "conferences of the parties," there have been a number of other important guidelines from WHO and the World Bank. In 2008, WHO released the strategy MPOWER: Monitor tobacco use and prevention policies, Protect people from tobacco smoke, Offer help to quit tobacco use, Warn about the dangers of tobacco, Enforce bans on tobacco advertising, promotion and sponsorship, Raise taxes on tobacco. The success of the adoption and implementation of MPOWER on national level was analyzed in later WHO reports, in particular on decreasing smoking prevalence and consumption and estimated smoking-attributable deaths (Ngo et al. 2017). As early as 1999, the World Bank published that tobacco tax should be increased to 80% of retailed price and that increase of tax and price, which is the most powerful tool to reduce smoking, should be supplemented by comprehensive advertising bans, smoke-free public places and work places, support of smoking cessation and intensive information on health effects including prominent warning labels (The World Bank 1999). Also later reports of the World Bank came to very similar conclusions.

In Europe, the Tobacco Control Scale of the European Cancer Leagues made use of recommendations by WHO and World Bank, granting a maximum score of 30 for a high average price of cigarettes (adjusted for purchasing power), a maximum score of 22 for smoke-free legislation and enforcement in work places, other public places and private cars, of 13 for comprehensive bans on direct and indirect advertising and promotion of tobacco products and cigarettes, of 10 each for spending on public information campaigns, for large pictorial health warnings with plain packaging, and for smoking cessation support. To reach a total maximum score of 100 also ratification of the Illicit Trade Protocol and compliance with its track and trace system (score 3) and enforced measures to restrict tobacco industry interference (score 2) were necessary (Joossens et al. 2020). Table 1 shows best ranks for United Kingdom, France, and Ireland, followed by Scandinavian countries. It has to be acknowledged, however, that tobacco control is more advanced in several countries outside of Europe, e.g., Australia, where tobacco prices are much higher, nicotine containing fluids are banned for ecigs, and smoking prevalence is much lower than in UK, especially in children. Australia introduced plain packaging in 2012 already.

In Europe, poor tobacco control and lowest progress was seen in Germany, still allowing tobacco advertising on billboards and in cinemas, and smoking is allowed in the hospitality industry in 11 of 14 federal states; in Switzerland hosting the headquarters of the international tobacco companies, probably the cause for the weak tobacco advertising legislation and why this country has not ratified the FCTC; and in Luxembourg, which has very low taxes on tobacco products, in order to attract cross-border shopping from neighboring countries. Prices of cigarettes adjusted for purchasing power are also too low in Central and Eastern Europe (Neuberger 2019a).

Progress in tobacco control was slower in Europe than in Australia or North America and average smoking prevalence is considerably higher. International comparisons of tobacco control legislations including country laws regulating ecigs and HTPs are provided, e.g., by the Johns Hopkins Bloomberg School of

Table 1 Ranking of Tobacco Control by European Cancer Leagues (Joossens et al. 2020). © Association of European Cancer Leagues. With kind permission

Ranking 2019 (ranking 2016)		Country	Price (30)	Public place bans (22)	Budget (10)	Ad bans (13)	Health warning (10)	Treat-ment (10)	Illicit trade (3)	Art 5.3 (2)	Total (100)
1 (1)	–	United Kingdom	25	22	0	12	9	9	2	1	80
2 (4)	▲	France	22	18	4	11	9	7	2	1	74
3 (2)	▼	Ireland	18	22	1	13	9	8	1	1	73
4 (3)	▼	Iceland	23	17	9	13	4	4	0	0	70
5 (5)	-	Norway	22	17	1	13	8	4	1	0	66
6 (6)	-	Finland	18	18	2	13	5	5	1	0	62
7 (new)		Israel	27	15	1	11	1	6	0	0	61
8 (28)	▲	Slovenia	12	16	2	13	9	6	1	0	59
8 (9)	▲	Hungary	15	21	0	11	5	6	1	-	59
10 (8)	▼	Spain	15	21	1	9	5	5	2	0	58
10 (17)	▲	Belgium	16	16	1	8	9	6	2	0	58
12 (7)	▼	Romania	16	21	0	8	5	6	1	0	57
13 (31)	▲	Greece	18	20	-	7	5	3	1	0	54
14 (9)	▼	Netherlands	14	15	1	9	5	7	1	1	53
15 (9)	▼	Sweden	14	15	0	9	5	7	2	0	52
15 (13)	▼	Italy	15	16	0	9	5	6	1	0	52
17 (9)	▼	Turkey	10	15	0	8	10	6	2	0	51
17 (13)	▼	Malta	16	12	0	11	5	5	2	-	51
17 (23)	▲	Croatia	16	11	0	12	5	5	2	-	51
20 (15)	▼	Portugal	18	11	-	10	5	4	2	0	50
20 (35)	▲	Austria	11	20	0	7	5	5	2	0	50
20 (17)	▼	Ukraine	17	15	-	11	4	3	0	0	50
23 (15)	▼	Poland	14	11	0	11	5	7	1	0	49
23 (26)	▲	Latvia	14	12	2	10	5	4	2	0	49
23 (31)	▲	Czechia	12	15	0	8	5	7	2	0	49
23 (21)	▼	Estonia	13	14	1	11	5	3	2	0	49
27 (19)	▼	Bulgaria	15	11	-	11	5	5	1	0	48
27 (26)	▼	Cyprus	15	10	0	11	5	5	2	-	48
29 (17)	▼	Russian Fed.	8	15	0	13	4	6	1	-	47
29 (28)	▼	Lithuania	12	13	1	10	5	4	2	0	47
29 (23)	▼	Denmark	13	11	2	8	5	7	1	0	47
32 (30)	▼	Slovakia	12	12	-	9	5	6	2	0	46
33 (23)	▼	Serbia	19	11	0	9	1	4	1	0	45
34 (33)	▼	Luxembourg	5	16	0	9	5	7	2	0	44
35 (21)	▼	Switzerland (-1)	13	11	4	2	5	7	0	0	41
36 (33)	▼	Germany	14	11	0	4	5	4	2	0	40

Public Health at https://globaltobaccocontrol.org. This site helps to keep up with rapid changes in laws and policies from across 98 countries. This policy bank has been verified by public health professionals in the respective country and is

separated into domains so that users can compare various national policies for minimum age, sale, marketing, packaging, product regulation, reporting, clean air, and taxation. Similar comparisons within Europe are made by the European Network for Smoking and Tobacco Prevention at http://ensp.network/. Binding regulations as given in the TPD can be found at https://ec.europa.eu/health/sites/health/files/tobacco/docs/dir_201440_en.pdf and a commentary on its revision at https://tobaccotactics.org/wiki/eu-tobacco-products-directive-revision/.

Even more diverse than the regulation of ecigs and HTPs is the legislation on other nicotine products. Oral tobacco has been banned in the EU except for Sweden, but oral nicotine has not been regulated yet. The problem with nicotine is that it is easily imported and that it can be mixed with just about anything. The historical mistake to sell tobacco products on the free market should not be repeated with other nicotine products. A positive example is Australia, which banned import of vape liquids with nicotine. For buying nicotine you need a prescription. In most countries, however, nicotine products are not subject to pharmaceutical law, but are sold without proper control of dose, additives, etc. and even provided in vending machines, which are not "child-safe" (Berger and Neuberger 2020), disregarding their high addictive potential.

The European Cancer Leagues in 2020 recommended 10 priorities to be fulfilled by all EU member states: to spend a minimum of €2 per capita per year on tobacco control, to implement at least the six World Bank priority measures in line with FCTC (Art. 4, comprehensive tobacco control policy), to address tobacco industry interference in public health policy making (Art. 5.3), to implement significant tax increases for cigarettes, hand rolled tobacco, etc. and a comprehensive smoke free legislation (Art. 8), including private cars carrying minors, to introduce standardized/plain packaging for all tobacco products, to ban the display of tobacco products at the point of sale, to accelerate implementation of tobacco cessation support (Art. 14), to ratify the FCTC Protocol to eliminate illicit trade and adopt tracking & tracing standards, and to invest in research to monitor and measure the effect of tobacco control policies (Art. 20).

The EU allows member states to go beyond the TPD (e.g., by requiring plain packaging) and also Art. 2.1 FCTC states "In order to better protect human health, Parties are encouraged to implement measures beyond those required by this Convention and its protocols, and nothing in these instruments shall prevent a Party from imposing stricter requirements that are consistent with their provisions and are in accordance with international law." Progress in tobacco control in some countries have led to plans of ending the tobacco epidemic completely, often called the "endgame." Usually the envisaged goal is to reduce smoking prevalence to below 5% in the population. Up to now only Bhutan banned tobacco sales and production, however, an increasing number of governments formulated long-term goals. In Finland a strategy was proposed to become not only smoke-free, but nicotine-free. Denmark's goal in 2016 was: "none of the children born today smoking in 2030." Australia, New Zealand, Sweden, Iceland, Norway, Ireland and multiple other nations might reach this goal in the next decade. Endgame strategies have to build on existing tobacco control measures that have proved effective. In countries where

tobacco industry and retailers still influence policy, the phasing out of cigarettes sales will take longer, but should be possible by applying the principles of consumer protection and human rights in a fair and equitable way to halt the sale of a deadly product (Smith and Malone 2019).

Cross-References

- ▶ Do Carcinogens Have a Threshold Dose? The Pros and Cons
- ▶ Epidemiological Methods in Regulatory Toxicology
- ▶ Examination of Organ Toxicity
- ▶ Extrapolation Procedures for Carcinogenic and Noncarcinogenic Compounds
- ▶ Nanoparticles and Their Regulation
- ▶ Psychoactive Designer Drugs: Classes, Mechanisms, and Regulation
- ▶ Specific Toxicity Tests for Neurotoxicity, Immunotoxicity, Allergy, Irritation, Reprotoxicity, and Carcinogenicity

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Regulation of Dental Materials

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Gottfried Schmalz and Franz-Xaver Reichl

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G. Schmalz
Department of Conservative Dentistry and Periodontology, University Hospital Regensburg, Regensburg, Germany

Department of Periodontology, University of Bern, Bern, Switzerland
e-mail: gottfried.schmalz@klinik.uni-regensburg.de

F.-X. Reichl (✉)
Department of Conservative Dentistry and Periodontology, University Hospital, LMU Munich, Germany and Walther-Straub-Institute of Pharmacology and Toxicology, LMU Munich, Munich, Germany
e-mail: reichl@lmu.de

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Abstract

Caries, periodontal diseases, and dental traumata belong worldwide to the most widespread diseases leading to tooth decay and missing teeth. Dental materials are playing a central role in treating these pathologies. Legal regulations concerning the safety of these materials were initiated in the 1970s of the last century (USA), and dental materials were classified as medical devices. This regulation was principally adopted by the EU in 1993 (Medical Device Directive, MDD) and revised in 2017 (Medical Device Regulation, MDR). International standards for assessing the safety have been developed for medical devices (ISO 10993 series) and also specifically for dental materials (ISO 7405). The latter respects the specific anatomical and physiological conditions in the oral cavity. Clinical risk assessment according to ISO 14971 determines the lacking information for a new material and defines the test strategy. Special attention was recently directed to nanomaterials, and the MDR has introduced a special classification rule for materials releasing nanoparticles. Dental materials need to be finalized intraorally, and thus dust is generated by grinding and polishing, but also by removing defective restorations. Worst-case calculations, however, revealed negligible exposure. Titanium (Ti) and zirconium (Zr) are preferentially used for endosseous dental implants, and the properties of Ti implants may be improved by coating them with nanostructured Ti-containing particles or Ti nanoparticles. However, it was claimed that periimplantitis can arise by exposure to TiO_2 particles, even in the absence of bacteria. Low release of Ti nanoparticles was found from dental Ti implants into the human mandibular bone, and therefore they might have no toxicologically clinical effects. Zr release from zirconia implants in animal mandibular bone was much lower compared to the Ti release from Ti implants. No histological abnormalities were found in animal tissues around zirconia implants.

In vivo tests for CMR and systemic toxic effects may be waved, if results from in vitro cytotoxicity/mutagenicity tests and chemical analyses of different material eluates in comparison with market products give no indication for such an effect. The TTC (Threshold of Toxicological Concern) according to ISO TS 21726 can be applied. Recently, EU regulations are directed to evaluate environmental aspects for dental materials, which may play an increasing role in the future.

Keywords

Resin composites · Nanoparticles · Nanomaterials · Titan · Zirconium · ISO standards · Medical Device Directive · Medical Device Regulation · Dentin · Dental pulp · Sensitization · Oral mucosa

Introduction

Diseases of the orofacial area include besides oral cancer and malformations mainly caries, periodontal diseases, and oro-dental traumata. Dental caries affects 2.5 billion adults (untreated caries) and 560 million children worldwide per year and is thus the most widespread non-communicable disease (Listl et al. 2015). According to the WHO, worldwide 15–20 percent of people aged 35–44 suffer from periodontal disease, an inflammation and infection of the gums. Its more serious form, periodontitis, if left untreated, often leads to an increased percentage of tooth loss. Globally, approximately 30 percent of people between ages 65 and 74 have no natural teeth remaining (Kassebaum et al. 2017; World Health Organization 2020). Oro-dental trauma is due to a number of issues, including unsafe schools and playgrounds, accidents, and violence, and between 16 and 40 percent of children aged 6–12 years old are affected by dental trauma (World Health Organization 2020).

The therapy of these pathologic conditions is heavily based on the use of dental materials, e.g., for restorations/fillings, crowns, and prostheses as well as of auxiliary tools like impression materials. To date regulatory safety regulations for these materials are meant to protect the patient, the dental personnel, and the environment. A special aspect for dental materials is the fact that many of them are delivered from industry in an unfinished state and that they are only finalized by the dentists; e.g., a resin paste is cured by using light curing units or by mixing with a separate catalyst paste, and then the cured material is adjusted intraorally by grinding and polishing.

The aims of this chapter are:

- To describe the development of regulatory safety regulations for dental materials.
- To address the specific regulations for dental materials.
- To discuss aspects typical for dental materials.

Development of Safety Regulations

History

Until the 1950s of the last century, in only few publications, the safety of dental materials was experimentally investigated: one of the first was the study from Fasoli about dental pulp reactions of dog teeth in response to the application of a filling material as early as 1924 (Fasoli 1924). In the 1950s and 1960s of the last century, the number of such animal-based studies increased, and the histological techniques as well as the evaluation procedures within involved experiments were improved (Schmalz 2002). Also at that time, cell culture techniques were first used to test the biological properties (here, cytotoxicity) of dental materials, which later developed into a major pillar of regulators biocompatibility evaluation of dental materials (Schmalz 2002). However, until the 1960s of the last century, no systematic approach on safety testing or safety assessment had been followed, and this field

was basically unregulated. Only by the beginning of the 1970s, the need for biological testing of dental materials was more and more advocated for, and the idea of standardization of such tests was born (Schmalz 2002).

Legal Framework

The first country to establish a legal framework regulating the safety of dental materials was the USA. In 1976 the "Medical Device Amendments" had passed the US Congress. While in the text of this regulation, the term "medical device" was used but not defined, later on in the Medical Device Directive (MDD), (European Union 1993) a medical device was characterized as any instrument, apparatus, appliance, material, or other article, which did not achieve its principal intended action in or on the human body by pharmacological, immunological, or metabolic means (for the full text of the current definition, see Medical Device Regulation (MDR) (European Union 2017a). Therefore, dental materials are classified as medical devices. Interestingly, the experiences from toxicity testing of dental materials in the USA had been one pillar for establishing this legislation (Schmalz 2002).

Within the European Union, the Medical Device Directive was adopted by the European Parliament in 1993 and became effective in 1998 (European Union 1993). As in the USA, also in Europe dental materials were classified as medical devices. This directive was constitutively revised and amended in 2017 and adopted as the Medical Device Regulation (MDR) in 2017 (European Union 2017a). One of the drivers for the tighter regulations of the MDR compared to the MDD was the breast implant scandal, where breast implants had been produced and sold by the company Poly Implant Prothèse (PIP), using illegally industrial-grade silicone since 2001. The MDR should become effective in spring 2020, but due to the coronavirus crisis in 2020, this had been postponed for 1 year (European Union 2020). Whereas the Medical Device Directive of 1993 had to be translated into national legislation of each EU country – leading to slight differences between EU member countries – the Medical Device Regulation of 2017 has become as such national law of all EU member countries. The abovementioned legal regulations define the general regulatory requirements for the safety and performance of medical devices including dental materials before being marketed. In the EU the manufacturer must show compliance ("Conformity") with the general safety and performance requirements laid down in the MDD or the MDR, and he is responsible for the safety of the medical device brought to the market by him. Depending on the risk class (see below), a third party ("Notified Body") is also involved for control.

Four risk classes have been defined within the MDD (European Union 1993) and the MDR, I, IIa, IIb, and III, taking into account the intended purpose of the devices and their inherent risks. From the dental material field, examples for the different classes are class I, paper points; class IIa, filling/restorative materials; and class IIb, materials for dental implants. Dental materials, which contain pharmaceutically active ingredients, are assigned to class III.

The assignment of new materials to the different risk classes is guided by a set of rules delineated in detail in the MDD/MDR. The conformity assessment procedure for class I devices should be carried out, as a general rule, under the sole responsibility of manufacturers in view of the low level of vulnerability associated with such devices. For class IIa, class IIb, and class III devices, an appropriate level of involvement of a third party ("Notified Body") is obligatory.

For class I dental materials, the manufacturer (whose quality management system is certified according to ISO 13485 Medical Devices) declares conformity with the relevant regulation (MDD or MDR) and keeps the technical documentation, which is only used in case of clinically overt adverse effect observed after clinical use. An exception is class I medical devices, which are placed on the market in sterile condition, have a measuring function, or are reusable surgical instruments; for these medical devices, these special properties require additionally the involvement of a notified body. For class II dental materials, the manufacturer completes the technical documentation and declares himself conformity with the relevant regulations, if he has installed a quality management system. The relevant technical documentations are subject to a regular audit by a notified body. For class III generally more stringent regulations apply. For instance, for class III devices, manufacturers should summarize the main safety and performance aspects of the device and the outcome of the clinical evaluation in a document that should be publicly available (MDR). Furthermore, the manufacturer shall lodge with the notified body an application for assessment of the technical documentation relating to the device which it plans to place on the market. For more details see Annex IX MDR (European Union 2017a). In the MDR a number of new classification rules were introduced; rule 19 especially addresses so-called nanomaterials and assigns them to the different risk classes, which for dental materials is of special relevance (see below).

Besides the abovementioned legal regulations, which directly refer to aspects of regulatory toxicology, a number of other legal regulations have been passed within the EU affecting more indirectly this area. One example is the EU CLP regulation, which has been developed for labelling and packaging of substances and mixtures (European Union 2008). While not legally binding, the symbols of the CLP regulation are also used for labelling of the medical devices like dental materials. Chemicals used for the production of dental materials have to comply with REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), which is a regulation of the European Union, adopted to improve the protection of human health and the environment from the risks that can be posed by chemicals (European Union 2006). However, under REACH, there is no obligation to register substances in amounts below 1 ton/year.

Outside of Europe similar legal regulations have been developed in the recent years. As was mentioned above, the Medical Device Amendments in the USA regulated the safety of dental materials, and the Food and Drug Administration (FDA) was made responsible for the process of classification and market approval. In contrast to the MDD/MDR, the FDA classifies according to three classes: class I, low risk, general controls [generally exempt from 510 (k)]; class II, moderate risk,

general controls and special controls [510 (k) generally required]; and class III, high risk, general controls and Premarket Approval (PMA) required and must demonstrate safety and effectiveness without relying on a predicate device. For dental materials, the most common pathway is the 510 (k) procedure (according to class II). Here, the substantial equivalence (SE) of a new product compared to a market product (predicate device) must be demonstrated. This substantial equivalence means that the new device being compared to a predicate device has:

- The same intended use as the predicate device.
- The same technological characteristics as the predicate device.
- Different technological characteristics, but does not raise new types of safety and effectiveness questions and is at least as safe and effective as the predicate device (Anusavice and Schmalz 2012; United States Food and Drug Adminitration 2014).

Development of Standardized Testing Procedures

The actual test methods and specific safety requirements to be fulfilled by each new dental material in order to demonstrate conformity with the general requirements of the MDD or MDR are not regulated by these legislations. However, the manufacturer can demonstrate the conformity with the abovementioned general legal requirements by testing their materials according to national/international standards or other technical specifications. Special reference within the EU legislation is made to "harmonized standards" (see below).

In 1972, the American Dental Association published recommendations on standard practices for biological evaluation of dental materials, which was then further developed and published in 1979 as ANSI/ADA document No. 41 (ANSI/ADA 1979). In Germany in the 1980s of the last century, the DIN 13930 was developed being mainly in line with the ANSI/ADA document No. 41 (Schmalz 2002). On an international level, the World Dental Federation (FDI) published in 1980 the FDI's "Recommended standard practices for biological evaluation of dental materials" (World Dental Federation 1980). This document was then further elaborated by the International Organization for Standardization (ISO) Technical Committee 106 (Dentistry) and published in 1997 as ISO 7405. The second edition was published in 2008 and the third in 2018 as "Dentistry—Evaluation of biocompatibility of medical devices used in dentistry" (ISO 7405 2018).

In the 1990s, the ISO Technical Committee 194 was founded, being in charge of developing standard testing procedures for the safety of medical devices. Since then a series of standards and technical specifications under the heading ISO 10993 was published by this committee (Table 1). Finally, "ISO 14971 – Medical devices—Application of risk management to medical devices" was published (ISO 14971 2019) describing the general procedures for performing a clinical risk assessment, which is required by the MMD/MDR. The clinical risk assessment according to ISO 14971 identifies missing necessary information on the safety of a new material. The general guidelines for selecting the specific test procedures to obtain this information

Table 1 ISO 10993 series of standards

Number: ISO 10993 part	Year	Title
1	2018	Evaluation and testing within a risk management process
2	2006, confirmed 2015	Animal welfare requirements
3	2014	Tests for genotoxicity, carcinogenicity, and reproductive toxicity
4	2017	Selection of tests for interactions with blood
5	2009, confirmed 2017	Tests for in vitro cytotoxicity
6	2016	Tests for local effects after implantation
7	2008, amended 2019	Ethylene oxide sterilization residuals – Amendment 1: Applicability of allowable limits for neonates and infants
8	2000, now withdrawn	Selection and qualification of reference materials for biological tests
9	2019	Framework for identification and quantification of potential degradation products
10	2010, confirmed 2016	Tests for irritation and skin sensitization
11	2017	Tests for systemic toxicity
12	2012	Sample preparation and reference materials
13	2010, confirmed 2019	Identification and quantification of degradation products from polymeric medical devices
14	2001, confirmed 2019	Identification and quantification of degradation products from ceramics
15	2019	Identification and quantification of degradation products from metals and alloys
16	2017	Toxicokinetic study design for degradation products and leachables
17	2002, confirmed 2016	Establishment of allowable limits for leachable substances
18	2020	Chemical characterization of medical device materials within a risk management process
TS[a] 10993–19	2020	Physico-chemical, morphological and topographical characterization of materials
TS 10993–20	2006	Principles and methods for immunotoxicology testing of medical devices
TR[b] 10,993–22	2017	Guidance on nanomaterials
23	Under development	Tests for irritation

[a]TS = technical specification
[b]TR = technical report

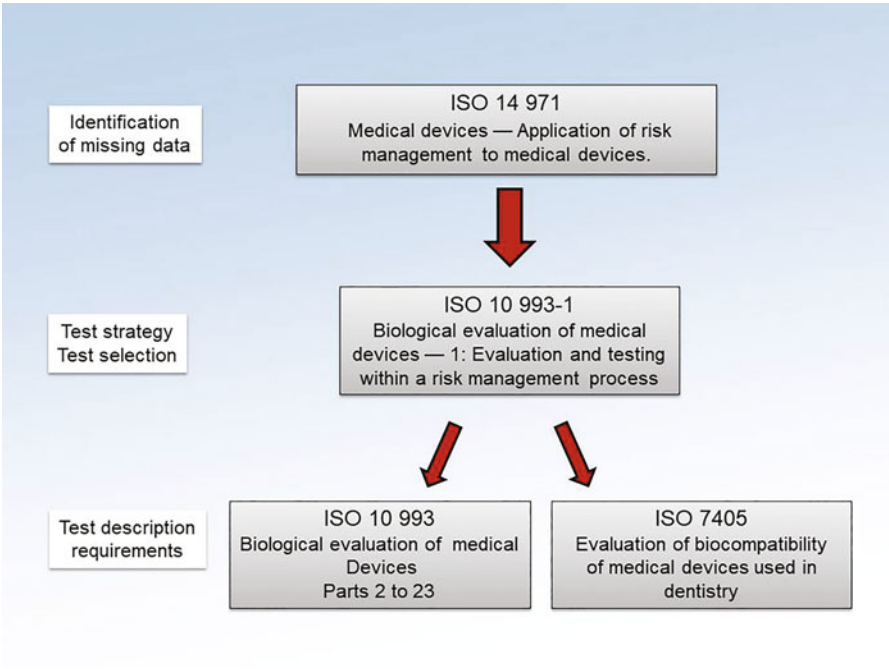

Fig. 1 The relation between the different standards used in the biocompatibility assessment according to the MDD or the MDR

are then described in ISO 10993-1 (ISO 10993-1 2018). The actual test descriptions are delineated in the following parts of ISO 10993 (Table 1) and in ISO 7405. The relation between the standards 14971, the ISO 10993 series, and the ISO 7405 is shown in Fig. 1. Within ISO 7405 three groups of tests are described:

- Group1: cytotoxicity tests including the dentin barrier test (see below); reference is also made to ISO 10993-5 (tests for in vitro cytotoxicity).
- Group 2: systemic toxicity, skin irritation and intracutaneous reactivity, delayed skin sensitization, genotoxicity, and implantation tests; here no procedures are described in ISO 7405, but reference is made to the relevant ISO 10993 standards.
- Group 3: dentally specific usage tests (see below).

In dentistry, international standards developed by ISO are automatically accepted by CEN (European Committee for Standardization) as European standards (EN standards) as part of the so-called Vienna Agreement (CEN CENELEC ISO 1991). Within the MDD and the MDR, especially compliance with so-called harmonized European (EN) standards as defined in Regulation (EU) No 1025/2012 (European Union 2012) should be a means for manufacturers to demonstrate conformity with the general safety and performance requirements ("presumption of conformity"). Such EN standards have passed an acceptance

procedure by the EU commission. For the MDD, a number of ISO dental standards for dental materials have been harmonized within the cumulative standard EN1641:2009 – Dentistry – Medical devices for dentistry, Materials (EN1641:2009 2009) containing also a reference to ISO 7405. Also ISO 10993 standards have been harmonized. Therefore, standards set up by international organization and by corresponding standards bodies have gained considerable importance. Within the new MDR, all standards harmonized so far within the frame of the MDD will become invalid. A new system of harmonized standards is assumed to be established by 2024. However, harmonized or not, standards are recognized as the state of the art for property testing (not only) the safety of dental materials.

Besides ISO standards, the OECD (Organisation for Economic Co-operation and Development) has issued so-called OECD guidelines for testing of chemicals assessing the potential effects of chemicals on human health and the environment. They are accepted internationally as standard methods for safety testing; they are continuously expanded and revised (https://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm). Some of these tests are also incorporated into abovementioned ISO standards and adjusted for testing medical devices.

Clinical Risk Assessment

The evaluation of the biocompatibility of (new) dental materials according to current regulatory toxicology concepts starts with a clinical risk assessment (Fig. 1). The procedure is described in detail in ISO 14971 (ISO 14971 2019). The process described in this document intends to assist manufacturers of medical devices to identify the hazards associated with the medical device, to estimate and evaluate the associated risks, to control these risks, and to monitor the effectiveness of the controls. This comprises:

- Risk analysis;
- Risk evaluation;
- Risk control;
- Production and post-production activities (Fig. 2).

Within the process of risk analysis and risk evaluation, it must be determined, if and which data are missing. Also dental materials have to be assigned to the relevant risk classes according to the classification rules, e.g., from the MDD or the MDR. Test selection and actual tests can be performed using ISO standards, for the EU also harmonized standards (see above). Then the final technical documentation with all necessary data is compiled as required by the MDD or MDR.

This whole system of a tiered regulatory approach defining responsibilities, testing strategies, test protocols, and control mechanisms according to the involved

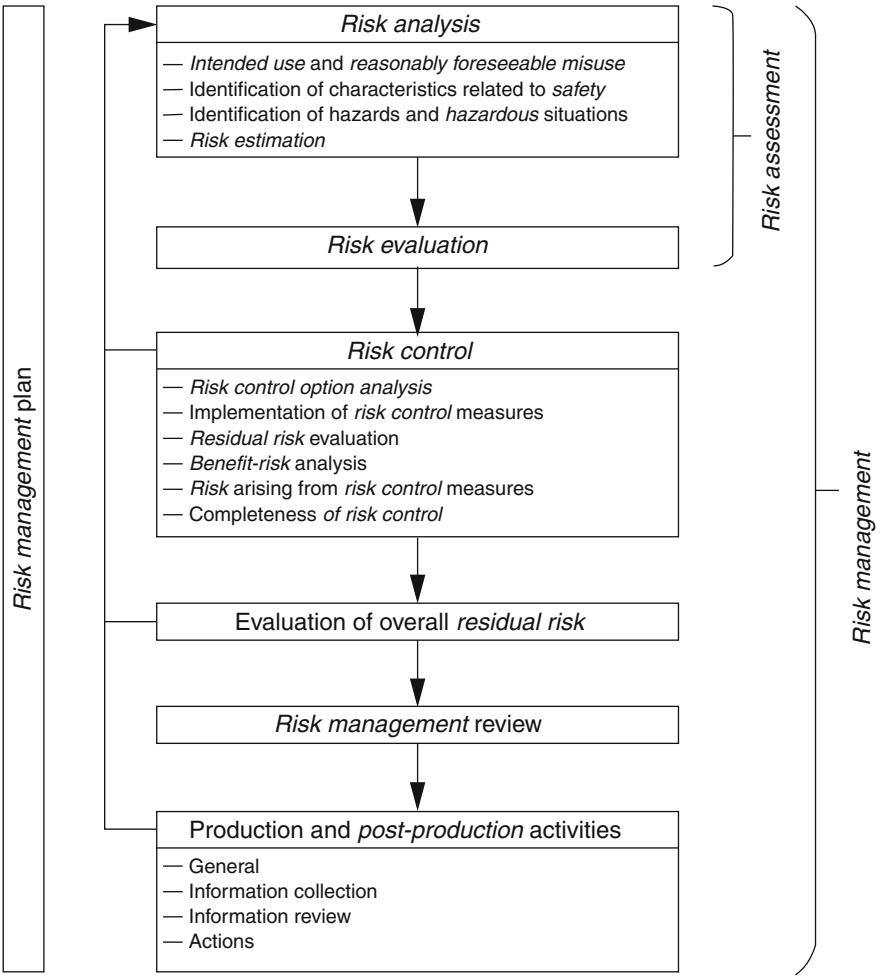

Fig. 2 Risk management scheme according to ISO 14971

risks has proven to be in most cases effective for dental materials since 1998. In the early phase of MDD implementation, new market products caused problem during clinical use (Braun et al. 2001). However, later on no major complications have become evident.

Unspecific Versus (Dentally) Specific Tests

In the abovementioned standards ISO 7405 and ISO 10993 series, so-called unspecific tests are described; additionally in ISO 7405 also dentally specific tests for safety evaluation are depicted. Unspecific tests are those, in their technical setup,

do not take the specific anatomical and physiological conditions in the oral cavity into account, whereas specific tests ("usage tests") do.

Unspecific tests are related to endpoints like cytotoxicity, genotoxicity/mutagenicity, or systemic toxicity. Tests are those described in the ISO 10993 series (Table 1) and – in part – in the ISO 7405, and they in some cases refer to relevant OECD guidelines (e.g., genotoxicity/mutagenicity tests, test for systemic toxicity after oral application). Many of these tests are in vitro tests or those using small laboratory animals, for instance, the subcutaneous implantation test in rats (ISO 10993-6) (ISO 10993-6 2016 2016). Extensive experience is available using unspecific tests for safety evaluation of dental materials (Schmalz et al. 2016; Schmalz and Galler 2017). Such tests are also an important part for demonstrating conformity with the general safety requirements of the MDD/MDR. While mechanistic approaches (Schweikl et al. 2006; Krifka et al. 2013) of dental materials have generally played no or only a minor role in regulatory toxicology, recently a test evaluating the anti-oxidative response of cells after exposure to (dental) materials has been suggested for being included as annex into ISO 7405 (antioxidant response element (ARE)) reporter assay.

Extrapolation of results derived from unspecific toxicity tests to the patient situation proved to be difficult in certain situations (Schuster et al. 2001; Schmalz et al. 2016). For instance, the restorative/filling material zinc oxide and eugenol, which is highly cytotoxic in standard cell culture tests, does histologically not show any damage on the dental pulp when applied in cavities of human teeth or those from experimental animals. The reason for this discrepancy is apparently that the specific anatomical and physiological situations of the tooth are not reflected in standard cytotoxicity test. The dentin between the cavity floor and the dental pulp seems to play a major role (Schmalz and Galler 2017) (Fig. 3).

The dentally specific tests, also named "usage" tests, are mainly animal-based tests and include according to ISO 7405 the:

- Pulp/dentin and pulp capping usage tests.
- Endodontic usage test.
- Endoseous dental implant usage test.

In general, dental materials are applied in these usage tests in a similar way as they are placed in patients; e.g., for testing dental restorative/filling materials, cavities are drilled in teeth of experimental animals like subhuman primates, dogs, or rats and then filled with the test materials and appropriate controls. Pulp reactions are evaluated histologically after up to 3 months in situ. A special test method is the "dentin barrier test", which is a cell culture-based method but simulates the anatomical situation in the tooth by interposing a dentin disk between the test material and the target cells ("simulation test"; see below) (Fig. 4) (Schuster et al. 2001; Schmalz and Galler 2017). All usage tests involve test animals like subhuman primates, dogs, or rats. These tests are costly and time-consuming and encounter ethical problems.

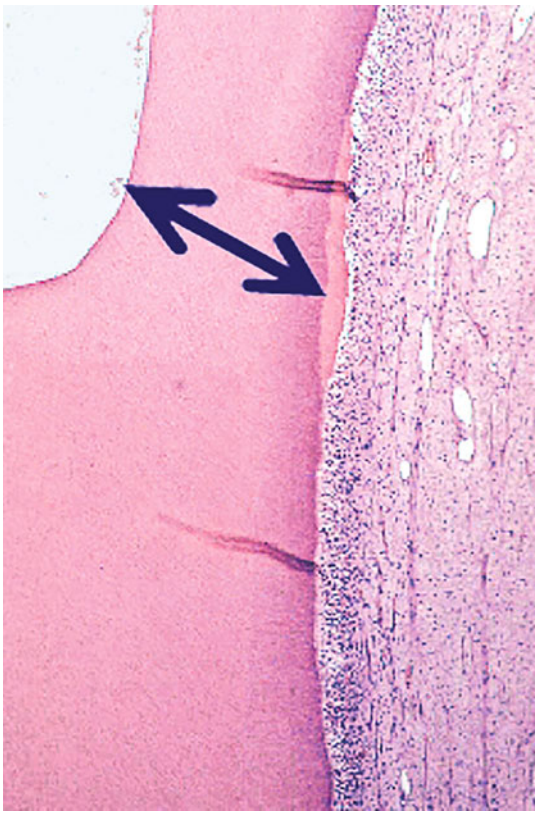

Fig. 3 Dentin between the restorative/filling material and the dental pulp may decrease the chemical damage upon the dental pulp (Schmalz and Arenholt-Bindslev 2009)

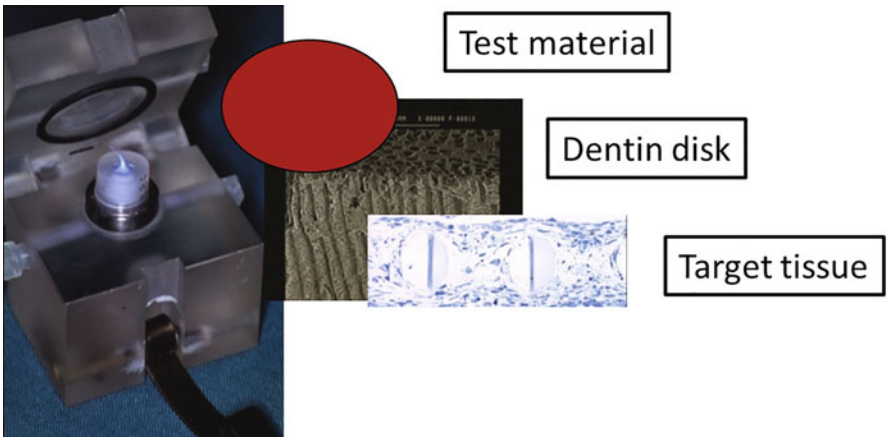

Fig. 4 Dentin barrier test, simulating the anatomical situation in the tooth by interposing a dentin disk between the test material and the target tissue (Schmalz and Arenholt-Bindslev 2009)

Specific Problems in Dentistry

Dental Pulp Damage

As has been delineated above, the dental pulp is an important target organ for the safety testing of dental (here, restorative/filling) materials. The chemically induced dental pulp damage due to the applied material results in inflammation and finally loss of pulp vitality (pulp necrosis). The standard test for evaluating a possible pulp damage according to ISO 7405 is the pulp/dentin usage test (see above). However, such a test involves the use of large test animals, which encompasses ethical and financial problems. Furthermore, the time frame for such a test including histologic evaluation is usually more than 1 year for one test series. Moreover, for such tests healthy teeth from test animals are used; in the clinical situation, however, teeth, which need restorations, have usually experienced caries or trauma, which both may lead to an already existing pulp inflammation. This then may add up to the chemically induced damage due to the restorative material. It is virtually not possible to reproducibly simulate such a cumulative effect in an experimental situation like in the pulp/dentin usage test. Therefore, a pulp inflammation as a consequence of material application in such tests is of clinical relevance; if no such pulp inflammation can be observed, this though is no guarantee that there will be no pulp damage in patients as a consequence of additive effects of a pre-exisitng inflammation and of the restorative material.

Therefore, the indication for this test must be considered very carefully. Today such a test is mainly recommended for dental restorative/filling materials with a complete new chemistry. For instance, some years ago, a complete new chemistry for restorative materials was introduced in order to reduce the material shrinkage during polymerization (Schweikl et al. 2002; Schmalz and Galler 2017). In contrast to the commonly applied acrylates, special epoxy compounds were used. In this case, the pulp-dentin test was justified. Other examples are materials which claim tissue regenerative properties, which presently cannot be evaluated in in vitro experimental setups. In most other cases, however, the pulp-dentin test can be waived, and data from cell culture tests according to ISO 7405 or ISO 10993-5 may be used instead. In comparing the cytotoxicity data of the new material with a market product (reference material), the expected reaction of the dental pulp can be estimated, if the chemical analyses of eluates are available. This is especially possible with materials with similar compositions as market products and if the mechanism for cytotoxicity is similar between test and reference material like a redox imbalance (Schweikl et al. 2006; Krifka et al. 2013).

An alternative to the pulp-dentin usage test is the abovementioned dentin barrier test (Fig. 4). Here, a dentin disk is placed between the test materials and the target cells, and thus the modifying influence of dentin acting as a partial diffusion and adsorption barrier is simulated. Furthermore, a three-dimensional cell culture has been developed based on immortalized dental pulp cells. This test has been adopted into the ISO 7405. For some dental materials, like those with a low pH (e.g., self-etching dental adhesives) or very hydrophobic materials (e.g., zinc oxide and

eugenol), which in classical cell culture test like those in ISO 10993 part 5 are cytotoxic, no cell reaction was seen in this dentin barrier test. This is in accordance with results after application of these materials in human teeth and those from experimental animals (Galler et al. 2005). For such tests the use of a standard positive (toxic) reference materials is necessary. Such a materials has been developed and has been included into the ISO 7405; it has produced reproducible in vitro toxic results in different laboratories (Schmalz et al. 2011)

Sensitization

Allergic reactions after exposure to dental materials have been observed in about 2% of dental personnel (Munksgaard et al. 1996). The frequency of such reactions in patients is less than 0.3% (Hensten-Pettersen 1992; Hensten-Pettersen 1998; European Commission 2015a), but the risk of sensitization has to be taken care of during the preclinical risk assessment. Mainly type IV reactions have been observed in patients, more seldom type I reactions (Schmalz and Arenholf-Bindslev 2009). The guinea-pig maximization test has largely been used for preclinical testing the sensitization potential of dental materials for type IV reactions (Schmalz and Arenholf-Bindslev 2009).This test is also adopted into ISO 10993-10 (ISO 10993-10 2016). Similar tests described for this purpose within ISO 10993-10 are the closed patch test (Buehler test), also performed in guinea pigs, and the murine local lymph node assay (LLNA). ISO 10993-10 is presently under revision with the aim to formulate two separate standards, one for sensitization (revision of ISO 10993-10, new title, tests for skin sensitization) and one for irritation (planned ISO 10993-23, test for irritation).

A technical problem of these tests is that the test substances should not be skin irritating. For some dental materials, this is a problem, because they may have a pH of 1 or even less (e.g., self-etching dental adhesives). Therefore, these materials/substances cannot be tested as such. Eluates can be used instead, and in preliminary tests it should be shown that the test substances are not skin irritating; otherwise they must be diluted.

Another problem is related to the fact that the abovementioned sensitization tests according to ISO 10993-10 evaluate skin sensitization. However, dental materials may have only skin contact with the user (dental personnel), but patients are generally having contact with their oral mucosa. Oral mucosa and skin differ in some aspects, e.g., the skin is keratinized, whereas the oral mucosa is mainly not. Thus the barrier effect may be different. Furthermore, materials in contact with the oral mucosa are also in contact with saliva, and this may dilute substances eluted from the test material. And finally, dendritic cells (DCs) are antigen-presenting cells that capture, process, and present antigens to lymphocytes to initiate and regulate the adaptive immune response (Reinartz et al. 2016; Song et al. 2018). They are present in the oral mucosa (especially Langerhans cells) and play a major role as both activators and silencers of allergic immune responses within the immunological network of mucosal surfaces (Novak et al. 2010). However, the composition of the

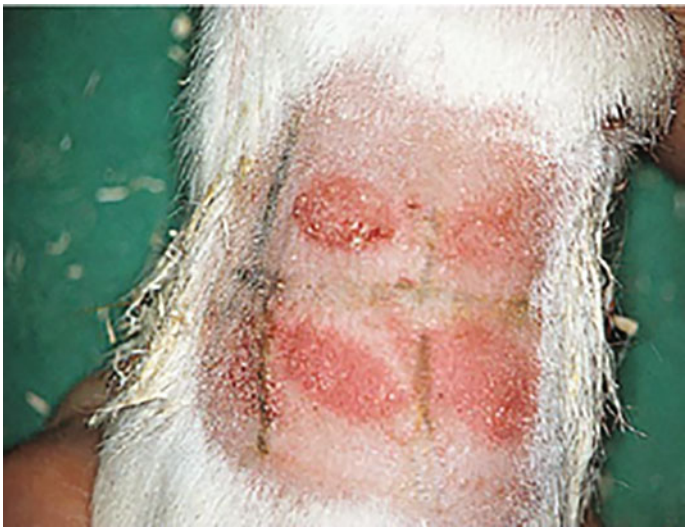

Fig. 5 Test results from the guinea maximization test (Schmalz and Arenholt-Bindslev 2009)

DC populations in different locations (e.g., dermis vs. mucosa) is different (Novak et al. 2010). Therefore, the relevance of skin testing for verifying the allergenic nature of the inflammatory response after oral application is under discussion. Contact allergy tests using the oral mucosa as target have been performed (epimucosal test) (Okamura et al. 2003). However, this test method proved to be less sensitive, and therefore today still the epidermal tests are recommended.

Recently, in vitro tests for preclinical evaluation of the different steps of sensitization have been developed and referred to in the annex to a working document ISO-DIS 10993–10 with reference to OECD 442C, OECD 442D, and OECD TG 442E. Experiences with such tests exist with pure substances; however little or no experience exists with testing medical devices or extracts from medical devices. Therefore, for the time being and for showing the conformity with the MDD/MDR, still animal-based methods are used, for dental materials preferably the guinea maximization test (Fig. 5).

Oral Mucosa

The oral mucosa is another target organ for dental materials. In patients, adverse reactions of the oral mucosa mainly comprise whitish strongly localized reactions limited to the contact area with the dental materials (local lichenoid reaction) (Fig. 6). The etiology of such reactions is yet unclear, but in more than 50% of such cases, a type IV allergy toward the contacting material could be shown (Schmalz and Arenholf-Bindslev 2009). Other factors may be localized repeated trauma of the oral mucosa due to rough material surfaces or sharp edges.

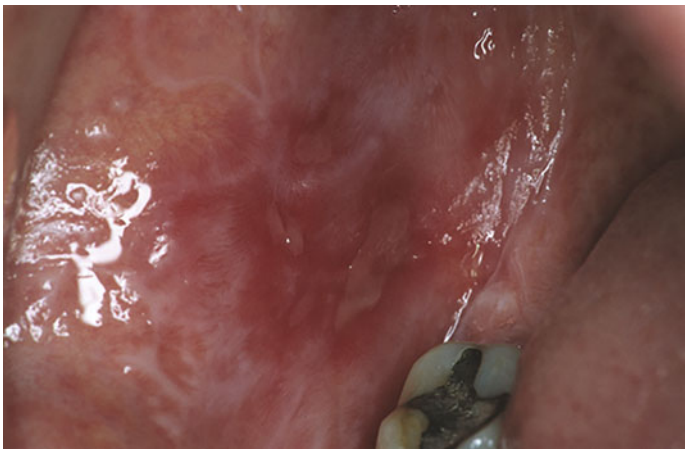

Fig. 6 Lichenoid reaction of the oral mucosa toward amalgam; patch test positive to mercury (Schmalz and Widbiller 2018)

For testing the effect on dental materials upon the oral mucosa, the hamster pouch test has been published (Harsanyi et al. 1991). A major problem with this test, however, was to keep the material in place for an appropriate amount of time (several weeks). Fixing the materials with sutures was not very effective, and the sutures themselves induced inflammatory reactions which superimposed those from the test material. Using a collar around the neck of the test animal was shown to be more effective (Harsanyi et al. 1991). However, such tests have not been adopted into toxicity testing standards for medical devices/dental materials, and experiences with tests like the hamster pouch test evaluating the mucosal reactions of dental materials are very limited. As a surrogate test, cell cultures are used according to ISO 7405 or ISO 10993-5, where the reaction of the test material is compared to market materials (reference material) with no clinical history of mucosa irritations.

Nanoparticles

Nanomaterials and Medical Devices

Nanoparticles having a size of 1 to 100 nm in at least one dimension are widely used in many products of daily life mainly to improve the performance, e.g., of materials or of drugs, but also of cosmetics, food stuff, and dietary supplements (Schmalz et al. 2017; Schmalz et al. 2018). Concerning regulatory toxicology, nanoparticles have recently gained increased public and scientific interest, and national and international agencies are dealing with safety aspects, e.g., WHO, ISO, EU Commission, or the FDA (Schmalz et al. 2017; Schmalz et al. 2018). In this context mainly the term "nanomaterial" is used.

According to the definition from the EU (2011), the term "nanomaterial" means a natural, incidental, or manufactured material containing particles, in an unbound state or

as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm. In specific cases and where warranted by concerns for the environment, health, safety, or competitiveness, the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50% (Schmalz et al. 2017; Schmalz et al. 2018). This definition of the EU is a recommendation and – according to the new EU Medical Device Regulation – a dynamic definition, which means that it can be changed, if new scientific information justifies it. However, so far, the above definition applies.

Rule 19 of the MDR deals especially with the risk classification of nanomaterials and defines:

- Class III if they present a high or medium potential for internal exposure;
- Class IIb if they present a low potential for internal exposure;
- Class IIa if they present a negligible potential for internal exposure (European Union 2017a).

Nanoparticles in Dental Materials

Nanoparticles play an important role in dental materials. They are intentionally added to dental materials, e.g., to resin-based composites, to improve technical properties like polishability and surface gloss. Typically, pyrogenic silica (SiO_2) is added, but also mixed oxides, such ZrO_2-SiO_2. Furthermore, nanoparticles are also unintentionally added. A large number of dental materials contain filler particles (mainly around several micrometers in size) in order to improve, e.g., mechanical properties, like in restorative materials, impression materials, or dental cements used for luting crowns. During the grinding process to generate such fillers, inevitably nanoparticles will result as by-products, even if the main size of the filler powder is intended to be in the micrometer area. The mass of this nanoparticle fraction is small, but the number of particles is comparatively high due to their small size. Therefore, it was estimated that more than 3500 dental materials fall under the EU definition of a nanomaterial (Schmalz et al. 2017; Schmalz et al. 2018).

As was mentioned above, dental materials in many cases are delivered in an unset state and are finalized (e.g., cured) by the dentist in the mouth of the patient. Then, the materials must be adjusted intraorally by grinding and polishing. During this process nanoparticles are released, even if the material itself does not contain nanoparticles (Bogdan et al. 2014). The same is true, if the material must be removed in the course of restoration replacement.

Nanoparticles are also intentionally placed on titanium implant surfaces. Such nanoparticles are strongly bound to the surface of the implant. The term “fixed” nanoparticles has also been used in the literature in this context. Nanoparticles on these surfaces are used for coating a medical device to prevent infection (e.g., silver nanoparticles) or to improve biocompatibility (e.g., apatite or Ti particles) (Schmalz et al. 2017; Schmalz et al. 2018).

This all demonstrates the importance of nanoparticles and of rule 19 of the MDR within the field of regulatory toxicology of dental materials. Finally, another consequence of this rule is that certain dental materials are now (MDR) assigned to a

higher-risk class than before under the MDD; for instance, dental impression materials, which were under the MDD in class I, are now under the MDR in class IIa. This all demonstrates the importance of a clinically relevant exposure assessment.

Nanoparticle Exposure from Dental Materials

According to rule 19 MDR, the internal exposure has to be evaluated. Although the term "internal exposure" has not been defined yet, the exposure in general has recently been estimated for the dental environment (Schmalz et al. 2017; Schmalz et al. 2018). Occupational exposure in the dental laboratory occurs due to the release of nanoparticles during handling of different materials like gypsum products and during grinding and polishing resinous materials, metals, or ceramics. Only if legal regulations for occupational safety are not followed, this may cause lung diseases (Schmalz et al. 2017; Schmalz et al. 2018). Occupational exposure in the dental office (dentist, dental auxiliaries) is of special relevance, because generally, dental personnel is considered to be a high-risk group for adverse effects of dental materials, due to their frequent and high exposure to dental materials. For premixed materials (pastes), an estimate of the nanoparticle exposure has revealed that virtually no nanoparticles are released from the nanomaterials. For materials, where a powder and a liquid are mixed, nanodust may develop during mixing, which may be inhaled. However, most powder/liquid materials are today delivered in capsules. Therefore, also for such materials, the exposure was estimated to be low to negligible (Schmalz et al. 2017; Schmalz et al. 2018).

Intraoral grinding and polishing of the set materials are often necessary, in order to adjust the applied material to the oral conditions like the adjustment to the opposing teeth (occlusion). Exposure has been estimated exemplarily for resin-based composites (Schmalz et al. 2017; Schmalz et al. 2018). Worst-case calculations were performed on a mass basis using data from Germany concerning the number of restorations annually placed and the number of dentists. The placement of ten restorations per day and a removal of 1 mm from the surface have been assumed. It was calculated that as a worst case grinding/polishing generates between 18 and 20 μg nanoparticles per day (Schmalz et al. 2017; Schmalz et al. 2018). This can be compared to the proposal of the German Agency for Occupational Safety (Bundesinstitut für Arbeitsschutz und Arbeitsmedizin) indicating a maximum acceptable nanodust concentration over a working day of 8 h and a concentration of 110–190 μg/m3 which corresponds to a daily exposure of 1100–1900 μg per day (Schmalz et al. 2017; Schmalz et al. 2018).

Exposure of patients to nanoparticles may occur during the application of the unset materials; but as was delineated for dental personnel, virtually no nanoparticles are released from unset resin-based composites (Schmalz et al. 2017; Schmalz et al. 2018). When set materials are adjusted intraorally, dust is produced, which is inhaled by patients. A similar worst-case scenario was formulated as for the exposure of dental personnel (see above) and the placement of five restorations/fillings per year

and the removal of 1 mm from the surface were assumed. An exposure of about 25 ng per day was calculated. For the wear of dental restorative materials during mastication, 50 µm and 100 µm surface loss per year was assumed based on the relevant literature. This resulted in a maximum mass loss of 133–266 µg per day, with only a minor part (<<0.1%) being nanoparticles. This can be compared to the normal daily exposure under unpolluted conditions, which was reported to be 400 mg nanoparticles per day (Terzano et al. 2010; Schmalz et al. 2017; Schmalz et al. 2018).

Titan (Ti) and Zirconium (Zr) Nanoparticles from Dental Implants

Titan (Ti) is one of the most biocompatible metallic materials because of its ability to form a stable and insoluble protective oxide layer (TiO_2) on its surface (Castilho et al. 2006; Elias et al. 2008). Ti and Zr are preferentially used for endosseous dental implants (Elias et al. 2008). It has been found that the properties of Ti implants can be improved by coating with nanostructured Ti-containing particles or Ti nanoparticles (Ti-NPs (NP = nanoparticles, the size is smaller than 100 nm)) (Valiev et al. 2008).

Even though Ti- and Zr-based implants are considered to be biocompatible, Ti-induced side effects such as hypersensitivity and allergic reactions have been reported/claimed (Lalor et al. 1991; Egusa et al. 2008; Sicilia et al. 2008). It has also been found that Ti-based materials may cause immuno-inflammatory reactions (Voggenreiter et al. 2003). These side effects might have been caused by the interaction between tissues and implants (Yang and Merritt 1994; Hansen 2008). Previous in vitro and in vivo studies showed that Ti ions and Zr can be released from Ti-/Zr-based implants, for example, by corrosion or wear (Woodman et al. 1984; Bianco et al. 1997; Jacobs et al. 1999; Browne and Gregson 2000; Okazaki and Gotoh 2005; He et al. 2020). The release of Ni ions from NiTi alloy also has been reported (Setcos et al. 2006).

A recent in vitro study (He et al. 2015) demonstrated a size-dependent cytotoxicity and DNA damage of Ti particles in periodontal ligament (PDL)-human telomerase reverse transcriptase (hTERT) cells. The EC_{50} values (representative for cytotoxicity) of investigated particles were 2.8 mg/mL (Ti-NPs <100 nm), 41.8 mg/mL (NiTi-MPs (MP = macroparticles, the size is bigger than 100 nm) <44 µm), and > 999 mg/mL (Ti-MPs <44 µm). Genotoxicity was described for Ti particles in the following range (Ti-NPs highest toxicity): Ti-MPs < NiTi-MPs < Ti-NPs (He et al. 2015). The highest cellular uptake efficiency was observed with Ti-NPs, followed by Ti-MPs and NiTi-MPs. Only Ti-NPs were found in the nucleus. Compared to Ti-MPs and NiTi-MPs, Ti-NPs induced higher cellular uptake efficiency and higher toxic potential in PDL-hTERT cells (He et al. 2015; He et al. 2020). Phagocytosis of Ti particles (<5 µm) could induce cytotoxicity in rat calvarial osteoblasts and MG63 human osteosarcoma cells (Pioletti et al. 1999; Lohmann et al. 2000). Genotoxic effects of Ti particles (<5 µm) have also been detected, which induced apoptosis in mesenchymal stem cells (Wang et al. 2002; Wang et al. 2003). It was found that the particles size can influence the toxicity of metal particles (Karlsson et al. 2009; Hackenberg et al. 2011). The ability of different particles

entering cells may also affect the toxicity (Karlsson et al. 2009; Oh et al. 2010; Hackenberg et al. 2011), and it is reported that particle size can impact the cellular uptake efficiency and pathway (Lee et al. 1993; Jiang et al. 2008; Zhu et al. 2013). Pettersson et al. (2017) found that Ti particles (and dental pathogenic germs) can activate the inflammasome-cascade reactions in macrophages and can increase the formation of cytokines. They concluded that periimplantitis can arise by titan oxide particles even in the absence of bacteria (Pettersson et al. 2017).

Previous studies reported that particles <1 μm could be taken up by non-phagocytic eukaryotic cells via endocytosis (Rejman et al. 2004) and particles with diameters exceeding 0.75 μm can be taken up by macrophages, neutrophils, and monocytes via phagocytosis or through macropinocytosis (>1 μm) by all cell types (Conner and Schmid 2003). Therefore, transportation of Ti particles with size of 0.5–5 μm into cells was observed in the human bone marrow tissues (He et al. 2016). Bone marrow fibrosis, avital bone tissues, and multinucleated cells were seen near dental implants (He et al. 2016). Bone marrow fibrosis might be induced by marrow injury and inflammation (Travlos 2006). These effects were reported to be associated with surgical trauma during insertion of implants (Piattelli et al. 1998). Multinucleated giant cells could be elicited by wear particles in periimplant tissues in human specimens, and these cells might contain phagocytized wear particles (Anazawa et al. 2004). Zr released from dental zirconia implants did not lead to abnormalities in animal bone marrow tissues (He et al. 2020).

Increased concentrations of metals (e.g., Ti, Cr, Co, and Al) derived from orthopedic implants in body fluids might induce acute or chronic toxicological effects (Hallab et al. 2001). The long-term effects of Ti and Zr derived from dental implants are still not fully understood, but associated hypersensitivity and allergic reactions in patients have been reported only for Ti in very few patients (Lalor et al. 1991; Sicilia et al. 2008). In a clinical study, the presence of titanium allergy was evaluated by the anamnesis and examination of patients, together with the selective use of cutaneous and epicutaneous testing, in patients treated with or intending to receive dental Ti/zirconia implants. In this study, 0.6% of 1500 patients were found to exhibit Ti allergic reactions (Sicilia et al. 2008). However, this has not yet been confirmed by other studies. Additionally, it has been found that detached metal debris from implants might cause marrow fibrosis, necrosis, and granulomatosis (Dannenmaier et al. 1985; Amstutz et al. 1992; Case et al. 1994). For the worst-case situation for patients with Ti implants, the following calculation can be made: the highest Ti content detected in human mandibular bone was 37,700 μg/kg bone (He et al. 2016). It is assumed that if all Ti in the bone is nano-Ti and 1 kg bone is 1 L fluid, a nano-Ti concentration can be calculated of 37 μg/mL. The EC50 value for nano-Ti in human cells is 2800 μg/mL (He et al. 2015). Therefore, it is assumed that Ti nanoparticles released from dental implants might have no toxicologically clinical effects. Zr release from zirconia implants in animal mandibular bone was much lower compared to the Ti release from Ti implants in human mandibular bone. No histological abnormalities were found in animal tissues with zirconia implants (He et al. 2020). From a clinical point of view, bacterial infection is still regarded to play a major role in eliciting/initiating a periimplantitis for both Ti and zirconia implants.

Protective Measures

Personnel protection, such as wearing a face mask against small particles, could be considered for protection against nanoparticles. Surgical masks with pore size of > 5 μm provided in a study quite some protection (64% for the respirable fraction of < 4 μm), taking into consideration that the normal dust exposure (i.e. without face mask) for dental personnel is low to negligible. However, as expected, FFP3 masks have a higher efficiency (72% for the respirable fraction of <4 μm), but they are cost intensive, complicate dental treatment (Breul et al. 2020) and not all persons can use them. FFP masks are certified according to EN 149:2001 + A1:2009 in class FFP2 and FFP3 and correspond to the indications of the World Health Organization (WHO) infection prevention and control guidance and according to the European Regulation (EU) 2016/425 (personal protective equipment) (World Health Organization 2019).

CMR and Systemic Toxicity

The term CMR refers to substances, which are carcinogenic, mutagenic, or toxic to reproduction. According to the new MDR, annex I, medical devices shall only contain CMR substances of category 1A (based on evidence from humans) or 1B (based on evidence from animal studies) in a concentration above 0.1% weight by weight (w/w), where especially justified and labeled. The classifications 1A and 1B follow the CLP regulation.

Carcinogenicity of Dental Materials

Certain substances, which are ingredients of dental materials, have shown under specific experimental condition carcinogenic properties, like nickel, beryllium, or cobalt (Wataha 2000; Geurtsen 2002; Schmalz and Garhammer 2002; Schmalz and Arenholf-Bindslev 2009). According to the International Agency for Research on Cancer (IARC), there is sufficient evidence in humans for the carcinogenicity of mixtures that include nickel compounds and nickel metal (International Agency for Research on Cancer 2018) and is assigned to group 1 (carcinogenic to humans), although for certain nickel compounds like nickel chloride, there is limited evidence in experimental animals. There is sufficient evidence in humans for the carcinogenicity of beryllium and beryllium compounds. Beryllium and beryllium compounds cause cancer of the lung. Beryllium and beryllium compounds are carcinogenic to humans (group 1) (International Agency for Research on Cancer 2018). According to the classification of the EU CLP regulation (European Union 2008), cobalt has recently been classified into category 1B (presumed to have carcinogenic potential for humans), and this classification was largely based on animal evidence.

However, in a recently issued literature compilation, the US FDA stated in 2019 for metal implants (including cobalt-chromium alloys) used in (orthopedic) surgery

that in summary, while isolated reports exist of cancers associated with metal implants, data from multiple large registries has failed to support any increased risk of malignancy with metal implants (United States Food and Drug Administration 2019). For dental materials there is no evidence from the dental literature that indicates that dental alloys are carcinogenic (Wataha 2000; Geurtsen 2002; Schmalz and Garhammer 2002; Schmalz and Arenholf-Bindslev 2009).

For certain acrylic monomers like TEGDMA or HEMA, which are used in certain resin-based restorative/filling materials and in dental adhesives, it has consistently been shown that they were mutagenic in different in vitro genotoxicity/mutagenicity tests. This was probably due to an induced redox imbalance (Schweikl et al. 2006; Krifka et al. 2013) or due to genotoxic/cancerogenic epoxy intermediates (Reichl et al. 2002). Again there is no evidence for carcinogenicity of dental resinous materials. And to the best knowledge of these authors, there have no reports in the literature been published demonstrating an association of oral cancer and applied dental materials in general. So, there is a discrepancy between mainly in vitro based data for genotoxicity/mutagenicity of dental material ingredients or eluates and the lack of clinical evidence for material-induced tumor formation.

As carcinogenicity tests using animal models are time-consuming and costly, carcinogenicity of dental materials is preclinically evaluated by in vitro genotoxicity/mutagenicity tests (see below) together with quantitative data from elution tests (amount of eluted substances) based on the TTC (Threshold of Toxicological Concern) concept according to ISO/TS 21726:2019.

Genotoxicity/Mutagenicity of Dental Materials

As mentioned above, genotoxicity/mutagenicity tests are mainly used as surrogate tests in the course of evaluating a possible carcinogenicity, and they should be considered during the clinical risk assessment. Genotoxicity tests are using mammalian or non-mammalian cells, bacteria, yeasts, fungi, or whole animals to determine whether gene mutations, changes in chromosome structure, or other DNA or gene changes are caused by the test samples (ISO 10993-3 2014). While genotoxic effects may be limited to a special cell generation, mutagenicity is referred to the fact that the genetic damage is transferred to the next cell generation.

Dental materials have elicited genotoxic effects, e.g., some root canal sealers (Schweikl et al. 1995; Ersev et al. 1999) in the bacterial reverse mutation assay (Ames test). Resin-based composite materials and the monomer components were genotoxic in the in vitro micro-nucleus test or the comet assay (Heil et al. 1996; Kleinsasser et al. 2004; Schweikl et al. 2006). However, this effect was mainly shown for the unset or very freshly mixed materials and for constituent monomers. Genotoxicity/mutagenicity tests, which generally are to be performed in the course of premarket evaluation of dental materials, are described in ISO 10993 part 3 (ISO 10993-3 2014) also with reference to relevant OECD tests. Modification of OECD tests for evaluating medical devices is described in in ISO/TR 10993-33 (2015).

According to ISO/TR 10993-33:2015 (2015), the following in vitro tests are recommended:

- Test for gene mutations in bacteria. Bacterial reverse mutation assay, OECD 471 technically modified for medical devices to allow, for example, testing with extracts from devices.
- And either:
  - An in vitro test with evaluation of chromosomal damage in mammalian cells. Chromosome aberration test, OECD 473 technically modified for medical devices.
  - An in vitro mouse lymphoma thymidine kinase assay, OECD 490 technically modified for medical devices including detection of small (slow-growing) and large colonies.
  - An in vitro mammalian cell micronucleus test for chromosomal damage and aneugenicity, OECD 487 technically modified for medical devices.

In vivo tests were included in the initial test battery of ISO 10993-3 in order to provide intact mammalian metabolism, and they have also been used to clarify results from in vitro tests. Currently, in vivo tests are not recommended generally for medical device assessment, but are reserved for rare circumstances (ISO/TR 10993-33:2015 2015). For dental materials, usually eluates are prepared, e.g., according to ISO 10993-12 (ISO 10993-12 2012), and the highest non-toxic/slightly toxic concentration is used for genotoxicity testing. If the results of the two in vitro tests are negative, further genotoxicity testing in animals is unnecessary (ISO 10993-3 2014).

Reproductive Toxicity of Dental Materials

Reproductive toxicity tests cover the areas of reproduction, fertility, and embryo-fetus development (ISO 10993-3 2014). These biological endpoints are also to be considered during clinical risk assessment. So far, no such effects have ever been reported for patients after exposure to dental materials. Tests for reproductive toxicity are described in ISO 10993-3. Reproductive and developmental toxicity testing is not required where an acceptable toxicological risk assessment of the medical device takes into account the fact that the risk of reproductive and developmental toxicity has been adequately mitigated (ISO 10993-3 2014). As for dental materials, such effects have not been clinically observed, these tests are generally waived. Again, data from standard cell culture tests and from the chemical analyses of material eluates can be used instead.

Systemic Toxicity of Dental Materials

For dental materials, systemic toxicity has mainly been a topic of concern for dental amalgam. In a 2015 report of the European Commission (SCENIHR), the more

recent literature on this topic has been compiled, and the conclusion was that the available data do not preclude the use of dental amalgam for the general population (European Commission 2015a). As a consequence of the Minamata Convention (see below), the EU has in 2017 enacted a regulation, by which dental amalgam should not be used in deciduous teeth, in pregnant and lactating women, and in children up to the age of 15 (European Union 2017b) except deemed necessary for medical reasons.

More recently, the effect of endocrine disruptors such as bisphenol A (BPA) has attracted increased interest, and BPA has been claimed to be responsible for many diseases like diabetes, obesity, or infertility (Talsness et al. 2009). In parallel to CMR substances, also endocrine disruptors have especially been mentioned in the new MDR, annex I, and again a concentration above 0.1% weight by weight (w/w) is only allowed, if specifically justified, and special labeling is required. Also dental restorative materials have been discussed in the context of BPA. Widely used resin composites contain resin bases like bis-GMA and similar compounds or (seldom) bis-DMA, and orthodontic bracket may be fabricated from polycarbonate resins. Although such dental materials do not deliberately contain BPA, it is a precursor of the abovementioned resins and thus is used in the production process (European Commission 2015b; Schmalz and Galler 2017). Residues of BPA are still present in the produced monomers in low quantities. It was shown that bis-GMA does not hydrolyze under physiologic conditions to BPA and bis-DMA does (Schmalz et al. 1999), but the abovementioned BPA residues are released (Sevkusic et al. 2014). However, concentrations of released BPA – even from bis-DMA-containing market products – are very low and beyond the derived temporary oral TDI of 4 μg/kg b.w./day (American Dental Association Council on Scientific Affairs 2014; European Food Safety Authority 2015; European Commission 2015b).

Tests for dental materials addressing acute, subacute, and chronic systemic toxicity are to be considered according to ISO 10993-1, depending on the exposure period of the medical device. Tests are described in ISO 10993-11, Biological evaluation of medical devices – Part 11: Tests for systemic toxicity (ISO 10993-11:2017 2017), which addresses the evaluation of unspecific systemic toxicity, not specific target organ or organ system toxicity. For dental materials oral application is the most reasonable route of administration.

Literature data from testing unspecific systemic toxicity as mentioned above indicate that dental materials do generally not elicit any acute systemic toxic reactions (Schmalz and Arenholf-Bindslev 2009). Such tests require the use of animals, and thus it has to be critically evaluated, if such tests are indicated for new dental materials. As recommended in ISO 10993-11 (ISO 10993-11:2017 2017), such tests for acute systemic toxicity may be waived, if cytotoxicity data are in the same range as for market products and if the chemical analysis of the eluates does not provide an indication for possible acute systemic toxic reactions. Basically the same is true for subacute, subchronic, and chronic systemic toxicity. Here the TTC concept can be applied, based on the total amount of extractable substances (see above).

Environment

Recently, the influence of dental materials on the environment has become a topic of interest, and thus scientific commission of the European Commission (SCHER) has issued an opinion of the impact of dental materials upon the environmento(European Commission 2014) (It was concluded that under conditions that amalgam separators are installed in the dental practices and the use of amalgam is low, no risk for the environment and secondarily for human health can be expected. This is the case for instance in Germany. The environmental risk for other restorative materials was assumed to be low, but there were no data for a sound risk assessment available. Such considerations are especially relevant when such materials are removed and discharged via the wastewater into the environment. The environmental impact of amalgam being discharged into the sewage water was also addressed in the Minamata Convention (United Nations Environmental Programme 2013), by which the use of mercury should worldwide be reduced due to environmental reasons. The consecutive EU regulation on mercury (European Union 2017b) has been mentioned above as well as to the restriction of amalgam use. Further to this, the discharge of amalgam waste into the environment has been regulated, and reference has been made to ISO 11143 for amalgam separators, which have to guarantee an efficiency (>95%) of amalgam particle removal (ISO 11143:2008 2008). Data for the environmental impact of other dental materials are missing, especially for resin-based composite materials. Here, the above mentioned release of subtances from discharged resin particles after restoration removal may be of interest due to a possible accumulation of such substances in the evironment. A recent in vitro study (Cokic et al. 2017) reported that relatively high concentrations of unpolymerized methacrylate monomers and of BPA were liberated from composite resin dust, which has been prepared by grinding a composite resin block. This demonstrates that further research is needed in this area.

Conclusions

Biocompatibility of dental materials is today tightly regulated by legal frameworks virtually all over the world, which are all based on the same principles, although they differ in details. The manufacturers are responsible for the safety and the performance of their products. They have to provide evidence (technical documentation) according to a scheme of different risk classes, and a third party is mainly involved in the medium- and high-risk classes (II and III) for control. Based on a risk assessment, missing data are identified, and relevant tests are mainly performed according to ISO standards. The changes introduced by the MDR in 2017 are in part due to problems which had been encountered with breast implants produced and sold by the company Poly Implant Prothèse (PIP), using illegally industrial-grade silicone since 2001. No experiences with the MDR are existing so far.

Cross-References

- ▶ Microplastics: A Novel Suite of Environmental Contaminants but Present for Decades
- ▶ Risk-Benefit Considerations in Toxicology
- ▶ Risk Characterization in Regulatory Toxicology
- ▶ Risk Cycles in Toxicology
- ▶ Risk Evaluation in Regulatory Toxicology
- ▶ Toxicological Risk Assessment
- ▶ Toxicological Risk Assessment in Different Jurisdictions

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Microplastics: A Novel Suite of Environmental Contaminants but Present for Decades

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Christian Laforsch, Anja F. R. M. Ramsperger, Simona Mondellini, and Tamara S. Galloway

Contents

Abstract

The ubiquitous contamination of the environment with plastic debris and the possible associated risks to ecosystems and, ultimately, human health has recently attracted a great deal of public and scientific attention. Among the plastic materials found in aquatic environments, microplastic particles have attracted particular attention since harmful effects on various organisms have been discussed, especially related to their ingestion. However, possible risks associated with microplastics cannot be generalized, as microplastics comprise a very heterogeneous group of particles that differ in their physicochemical properties. At present, there is a considerable lack of knowledge on the effects of microplastics at the molecular, cellular, tissue-specific, and organismic levels and the

C. Laforsch (✉) · A. F. R. M. Ramsperger · S. Mondellini
Animal Ecology I and BayCEER, University of Bayreuth, Bayreuth, Germany
e-mail: christian.laforsch@uni-bayreuth.de; anja.ramsperger@uni-bayreuth.de; simona.mondellini@uni-bayreuth.de

T. S. Galloway
College of Life and Environmental Sciences: Biosciences, University of Exeter, Exeter, UK
e-mail: T.S.Galloway@exeter.ac.uk

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resulting consequences on environmental and human health. This chapter addresses the benefits of plastic products but also why plastic has turned into an environmental problem. It briefly explains how environmental contamination is assessed and shows on which biological levels potential harmful effects are expected.

Keywords

Plastic debris · Microplastics · Nanoplastics · Environmental risks · Tissue translocation

Introduction

Modern Plastics: A Success Story Turned into an Environmental Problem

Plastics have become essential components of our everyday life and have made a wealth of technical and medical innovations possible (Andrady and Neal 2009). Plastic products are light yet stable and corrosion-resistant and have excellent insulating properties, to name only a few of their advantages. Due to the versatile material properties and the low production costs compared to other materials, plastics can be found in a variety of products. For instance, plastic packaging reduces food waste by extending shelf life. Plastics play a central role in the lightweight construction of vehicles, ensuring that they consume less fuel and reduce greenhouse gas emissions such as carbon dioxide (CO_2). In freight transportation over long distances, fewer greenhouse gases are emitted with the use of plastic products compared to alternative heavier materials like glass. Plastics are also used in the insulation of houses to reduce energy consumption, and they play a crucial role in the construction sector as pipes and cables, cladding, seals, adhesives, and gaskets. Plastic is essential in medicine, for instance, to ensure the sterility of medical products, and without plastic, neither computers nor smartphones would make our lives easier. Although there are a huge variety of plastics, the majority of plastics processed are limited to only a few types: polyethylene, polypropylene, polyvinyl chloride, polyurethane, polyethylene terephthalate, and polystyrene.

Since the 1950s, plastics' global production has risen from 1.5 million tons to 359 million tons in 2018, with production rates forecast to continue to rise sharply, doubling in 20 years (Plastics – the Facts 2019). China has the largest share of world production with 25%, followed by the European Union with 20% and North America with 19%. Among the main applications for plastics (~40%) are short-lived disposable products in the packaging industry. Correspondingly, the amount of plastic waste produced has also risen rapidly over the years. The proportion of plastics going into the recycling process was estimated to be only 31% in Europe in 2016. The remainder of plastic waste continues to be dumped in landfills across

Europe or sent for other forms of exploitation, such as incineration (Plastics – the Facts 2017). However, it can be assumed that in developing countries in particular, which often lack a proper collection system, the proportion of recycled plastic is far lower than in Europe.

Unfortunately, a significant proportion of plastic waste is released into the environment through careless and improper disposal (Browne et al. 2011; Dubaish and Liebezeit 2013). Especially this improper disposal of plastic waste inevitably leads to a long-term environmental problem. That is why plastic has changed from being a cheap problem-solver to an environmental problem itself.

The World Economic Forum has calculated, for example, that every year, approximately 32% of plastic packaging material alone is improperly disposed of in the environment (World Economic Forum 2016). The main problem of plastics in the environment arises from the high resistance and durability of the material. Due to the slow degradation dynamics, it is assumed that many plastics are persistent in the environment for hundreds of years, depending on the polymer type (Barnes et al. 2009). Therefore, it can be assumed that most of the plastic that has been released into the environment since the beginning of mass production is still to be found there and represents far more than just an aesthetic problem.

Once released into the environment, plastic disintegrates over time into ever-smaller particles due to weathering processes. Fragmentation occurs due to various environmental influences such as solar radiation and chemical and biological degradation. As a result, the material becomes cracked and brittle and continues to break up due to mechanical effects such as wave movements. The resulting particles are referred to as microplastics **(**Fig. 1**)**.

Fig. 1 Microplastics: Different kinds of microplastics found on the shoreline of Lanzarote. (© Christian Laforsch, University of Bayreuth)

Microplastics: A Young Field of Research

The term "microplastics" refers to fragments, fibers, films, foams, and spherical particles of plastics smaller than 5 mm. Accordingly, this definition covers a wide range of materials that, due to their specific chemical and physical properties, are likely to exhibit different behaviors and effects in the environment. Till now, there is no consensus on the definition of the actual size range of microplastic particles. The upper size limit of 5 mm is widely accepted, whereas the lower size limit is still under debate. The suggestions of the lower size limit range from 100 μm down to 100 nm, depending on the scientific field. For instance, for analytical studies of microplastics in the environment, the definition of the lower size limit often refers to the technological detection limitations, whereas for toxicological studies, lower size limits are proposed, as these may promote its bioavailability under laboratory conditions (Wright et al. 2013b; Frias and Nash 2019). Furthermore, there is no consensus on the definition of nanoplastics because it depends on the lower size limit of microplastics. However, an overall accepted distinction is made between "primary microplastics" and "secondary microplastics." "Primary microplastic" is manufactured industrially as a component of, for example, cosmetics, cleaning products, or abrasives and is discharged into the environment mainly via the wastewater (sewage treatment plants, rainwater, and combined sewerage systems). "Secondary microplastic" results from mechanical, chemical, and/or biological degradation from large waste fragments (macroplastics) or an abrasion from various plastic products (e.g., agriculture, construction industry, traffic, clothing) and can enter the environment in large quantities via various input paths. One example is tire abrasion from motor vehicles, consisting mainly of polyisoprene in the form of tiny particles. It is now considered certain that microplastics occur worldwide in all habitats, in some cases in considerable quantities.

Although the occurrence of microplastic in marine systems was reported as early as 1972 (Carpenter and Smith 1972), it took over 30 years until it became a hot topic with the publication of Thompson et al. (Thompson 2004) "Lost at Sea: Where Is All the Plastic?". Since then, microplastic has been detected in marine ecosystems worldwide and classified as a potential threat to biota, economy, and society **(**Fig. 2**)**. Although 50–80% of the waste found in the sea is produced and disposed of on land until recently, research has focused mainly on the supposed main sink of plastic waste, the ocean, where significant amounts of plastic waste are floating on the surface (Eriksen et al. 2014). Extrapolations indicate that between 1.1 and 12.7 million tons of plastic waste are discharged into the oceans via rivers worldwide each year (Jambeck et al. 2015; Lebreton et al. 2017). Despite a large number of publications on the marine system's microplastic contamination, there is still a lack of decisive information, for example, on the spatial distribution of microplastic in the oceans. Nevertheless, microplastics are reported to occur from tropical to pristine polar areas and from beaches to deep-sea sediments.

Freshwater ecosystems such as rivers and lakes have more recently received attention, and plastic particles have been found in areas used by tourists and even in remote mountain lakes (Imhof et al. 2013; Dris et al. 2015). Considering that the

Fig. 2 Beach on Lanzarote. Visible contamination with large microplastic fragments. (© Christian Laforsch, University of Bayreuth)

majority of plastic waste is generated and emitted on land, it is not astonishing that plastic particles have only recently been found in the atmosphere and terrestrial ecosystems, especially in urban and agricultural soils (Fig. 3) (Dris et al. 2016; Piehl et al. 2018; Weithmann et al. 2018).

Studies show that limnetic and terrestrial systems can serve, as well as marine systems, as sinks of plastic waste. No clear correlation could be established between the occurrence of microplastic in the environment and population density and

Fig. 3 Plastic on agricultural soils. All larger fragments can disintegrate into microplastics over time. (With kind permission from © Stefan Leible (University of Bayreuth))

proximity of industrial plants, which underlines both the complexity of the issue and the need to understand the mechanisms of the environmental behavior of microplastic. An estimate of the abundance of microplastic on agricultural land suggests that the pollution in Europe and North America alone is higher than the total amount of microplastics in the oceans (Nizzetto et al. 2016). Recent estimates suggest that there is almost 40 times more macro- and microplastics on land than in the ocean (Kawecki and Nowack 2019). The latter underlines the fact that plastic contamination of the environment not only affects the world's oceans but is of global relevance as a terrestrial pollutant.

It is predicted that, as a result of global population growth, increasing urbanization, and rising consumption in developing and emerging countries, the production and consumption of plastics will continue to grow strongly on a global scale and that the problem of the entry of microplastic into the environment will, therefore, become increasingly important. The resulting publicly discussed need for action is enormous, from which an urgent need for research can be derived to close the considerable gaps in knowledge that are becoming increasingly apparent despite or because of the topicality of the issue.

According to current estimates, "secondary microplastic" represents the main component of environmental contamination by microplastics. However, the extent of the contamination of the environment cannot yet be fully determined. Microplastics in the environment, unlike soluble pollutants, are neither temporally nor spatially homogeneously distributed, and therefore most studies on microplastic contamination are snapshots in time. In addition, microplastic analysis methods are only just being developed, as it is far from trivial to isolate and analyze microplastic particles smaller than the diameter of a human hair from complex

environmental samples. Particles in the nanometer size range have not yet been detected in the environment. Further, data on contamination of the environment with microplastics are often not comparable with each other, as no uniform methodology for the detection of microplastics has yet been established, and different methods are used for sample processing and analysis.

Microplastics: Analysis of Environmental Concentrations

Representative sampling is among the most critical step in the analysis of microplastics. Non-representative sampling leads to unreliable data, regardless of how reliable the subsequent sample processing and analysis is. Each sampling design must be adapted to the specific research question.

To chemically identify and quantify each plastic particle occurring in environmental samples, potential microplastic must be extracted from the sample volume. Usually, an environmental sample contains more natural particles in the form of plant, animal, and mineral constituents than microplastic. Water samples usually contain only a few mineral particles and a high proportion of organic material. For non-homogeneous solid samples such as soils, microplastic isolation is even more challenging. The difficulty in the purification of the samples is to avoid method-related damage or fragmentation of the microplastic particles as far as possible. The simplest method for microplastic isolation is sieving and manual sorting using a stereomicroscope. This method is not only limited to sizes >500 μm but is also very susceptible to misidentifications and observer bias, so a subsequent reliable polymer identification is essential.

Suggested methods for removing the mineral fraction include electrostatic separation, oil extraction, froth flotation, magnetic extraction, vertical density gradient separation, and density separation. Usually, saturated salt solutions of sodium chloride, zinc(II) chloride, or sodium polytungstate are used. A density of 1.6–1.8 g/mL is suitable for all environmentally relevant plastics. Various methods have been developed based on the principle of density separation for sample preparation, such as the Munich Plastic Sediment Separator (MPSS) (Imhof et al. 2012).

For the removal of the organic fraction, the use of strong acids and bases has proven to be unsuitable, as some polymer types can be strongly damaged or even completely dissolved. Small microplastics and fibers are particularly affected. The treatment with various technical enzymes combined with mild oxidizing agents such as hydrogen peroxide has proved to be a gentle method of extracting microplastic from environmental samples (Löder et al. 2017).

The pretreated samples are then applied to filters for the chemical analysis. Raman spectroscopy and Fourier transform infrared (FTIR) spectroscopy are the most commonly used state-of-the-art analytical methods in microplastic research. Both vibrational spectroscopy techniques enable the precise identification of polymer types and their abundance, shape, and size. Software-based automatic detection of microplastics has been developed (Fig. 4) (Hufnagl et al. 2019). However, next to

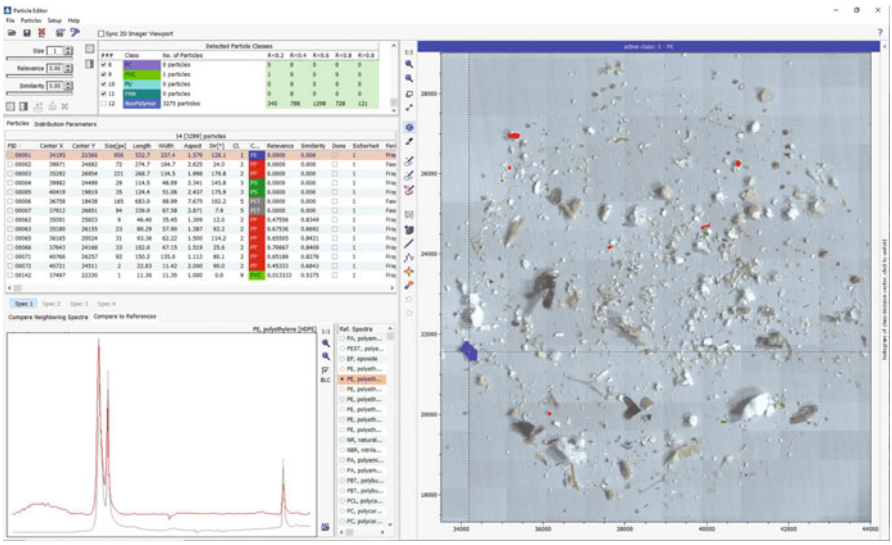

Fig. 4 Software-based automatic detection of microplastics using focal plane array (FPA)-FTIR (Hufnagl et al. 2019). Right-hand side: optical image of a filter after sample processing. The blue-labeled particle is identified as polyethylene (PE). Left-hand side: respective fingerprint spectra of the polyethylene particle. (© Martin G. J. Löder and Christian Laforsch, University of Bayreuth)

the restricted size limit of the respective methods (10 μm for FTIR, 300 nm for Raman), the spectra of environmentally aged plastics cannot always be clearly identified because biofilms on the particle surfaces can interfere with the spectroscopic methods.

If the shape and size of microplastic, which is indispensable if the toxicity on organisms is considered, are not in the focus of the study, microplastic can further be analyzed using pyrolysis gas chromatography mass spectrometry (Pyr GC-MS) or thermal extraction desorption gas chromatography mass spectrometry (TED GC-MS). With these methods, the polymer components, as well as the contained additives, can be examined under defined thermal conditions. However, these methods are restricted in the sample volume, which can be used (Fries et al. 2013; Dümichen et al. 2017).

In general, during sampling, sample processing, and analysis of microplastics, it should be noted that the risk of contamination of environmental samples is very high, as synthetic polymers are ubiquitous. Therefore, precautions must be taken at each processing step: blank samples should be used at each step, and plastic material should be avoided and replaced by alternative materials such as metal or glass wherever possible.

Woodal et al. (2015) comprehensively describe the application of a forensic-scientific approach to minimize sample contamination. A comprehensive discussion on the advantages and disadvantages of further and all described methods are listed in Möller et al. (2020).

Microplastics: Environmental Risks

The ubiquitous contamination of the environment with microplastics and the possible associated risks to ecosystems and ultimately to human health has recently attracted a great deal of public and scientific attention. Potential biological risks of plastic particles in the environment arise from the small size of the particles through which it can easily enter the food chain, mistaken as food or by inhalation. Microplastic particles can further degrade into even smaller particles, which have been termed "nanoplastics." Nanoplastics, with their smaller sizes, may have a higher bioavailability than microplastics and may even pose a higher environmental risk. Contamination of the environment with plastic particles, therefore, represents a global challenge and is (G7 Summit2015) classified as a "top emerging global issue" due to the as yet unassessed hazard potential and ubiquitous occurrence (GESAMP 2015).

The ingestion of plastic particles, together with natural food, has already been investigated in various organisms from aquatic and terrestrial habitats. The resulting effects on organisms and human health are still under discussion. It has to be noted that possible risks associated with plastic particles cannot be generalized since micro−/nanoplastics comprise a very heterogeneous group of particles that vary in polymer composition, additive content, size, shape, aging state, and, consequently, their physicochemical properties.

Hence, microplastic is only a collective term for small particles (< 5 mm) of various types of plastics with a wide range of chemical and physical properties as well as different surface conditions (e.g., functional groups, zeta potential). Non-polymerized monomers, as well as adsorbed organic material and coating with biomolecules and inorganic substances, contribute to the further complexity of the particles. Overly broad generalization over the potential biological effects predicted for microplastic is hence of limited value.

A central bottleneck in the assessment of the environmental relevance of microplastic is the lack of comprehensive data on biological mechanisms of action of microplastics as a function of the chemical and physical properties of the various plastics degraded in the environment. A comparison of microplastic with naturally occurring particulate materials, which can also be ingested with food, is indispensable. Considerable knowledge deficits currently exist at the molecular, cellular, tissue-specific, and organismic levels and the resulting ecological consequences.

Next to direct effects on organisms, which are discussed in the following paragraphs, plastic debris could also exert indirect effects in the environment leading to ecological consequences. Plastic debris could, for instance, act as a substrate and transport vector for alien species (Rech et al. 2016). Further, Trotter et al. (2019) found the interspecific communication between predator and its prey being interfered by the sole occurrence of plastic in the aquatic environment. The authors assume that the allelochemicals used for communication may adsorb to the surface of the plastic particles. The resulting misperception of the chemical cues may lead to a false adjustment of the prey's defensive strategies and may, therefore, affect population dynamics in higher orders of the food web.

To date, most studies about the effects of microplastic pollution mainly focused on the direct effects resulting from exposure. In particular, physical effects like injuries due to entanglement can cause severe inflammation of the affected tissue. Other possible consequences are drowning due to reduced mobility.

Moreover, if plastic particles are mistaken with natural food, the stomach capacity can be reduced, or the stomach passage blocked, which can lead to a false sense of fullness and, in turn, to a slow death from starvation.

Ingestion: The Main Entrance Route of Microplastics into Organisms

The degradation of plastic particles into smaller and smaller fragments increasingly affects organisms at lower trophic levels. Microplastics can float on the surface of water bodies, disperse in the water column, or accumulate in the sediment, making them accessible to a wide array of organisms in different habitats. Hence, the ingestion of plastic particles together with natural food has already been investigated in a variety of organisms, ranging from low trophic levels like zooplankton and mussels right up to higher trophic levels like vertebrates, from aquatic and terrestrial habitats. Several laboratory experiments, mainly conducted with aquatic organisms (including ciliates, cnidarians, rotifers, annelids, copepods, cladocerans, amphipods, mysids, euphausiids, barnacles, mussels, tunicates, and fishes), confirmed microplastic ingestion and uptake across the gill (e.g., Duis and Coors 2016). Some studies have already been carried out with terrestrial organisms, and ingestion was confirmed, for example, in detritivore soil invertebrates (Zhu et al. 2019). The ingestion of microplastic particles is additionally enhanced for microplastic being environmentally aged (Hodgson et al. 2018; Vroom et al. 2017). Moreover, some studies suggest that the presence of a microbial biofilm makes the microplastics more palatable for those organisms (Helmberger et al. 2019).

Upon ingestions, laboratory experiments suggest the excretion of microplastic particles within hours or days (Duis and Coors 2016), although the knowledge about microplastics retention time and excretion is still scarce. Some studies carried out with the Mediterranean mussels (*Mytilus galloprovincialis*) had conflicting results. Kinjo et al. (2019) reported that 99% of the ingested microplastics were excreted within 2 days after exposure. This study pointed out that very small microplastics were excreted faster than the larger ones. Those were detected in the feces up to 40 days after the exposure. Opposite results were obtained by Fernández and Albentosa (2019), as their work suggested that larger particles are excreted faster from the intestinal tract than the smaller ones. These contrary results are likely due to the different size range used in the two independent studies.

An investigation carried out on fathead minnows (*Pimephales promelas*) described excretion occurring within hours of exposure and dependent on the microplastics' size, with again the larger particles being excreted faster (Hoang and Felix-Kim 2020). The excreted microplastics were left available for reconsumption and were detected within the gut at all monitoring points. The excreted microplastics were coated with intestinal liquids, resulting in their aggregation and precipitation to the bottom of the exposure beakers. This suggests that the excretion process may contribute to the vertical movement of microplastics from the

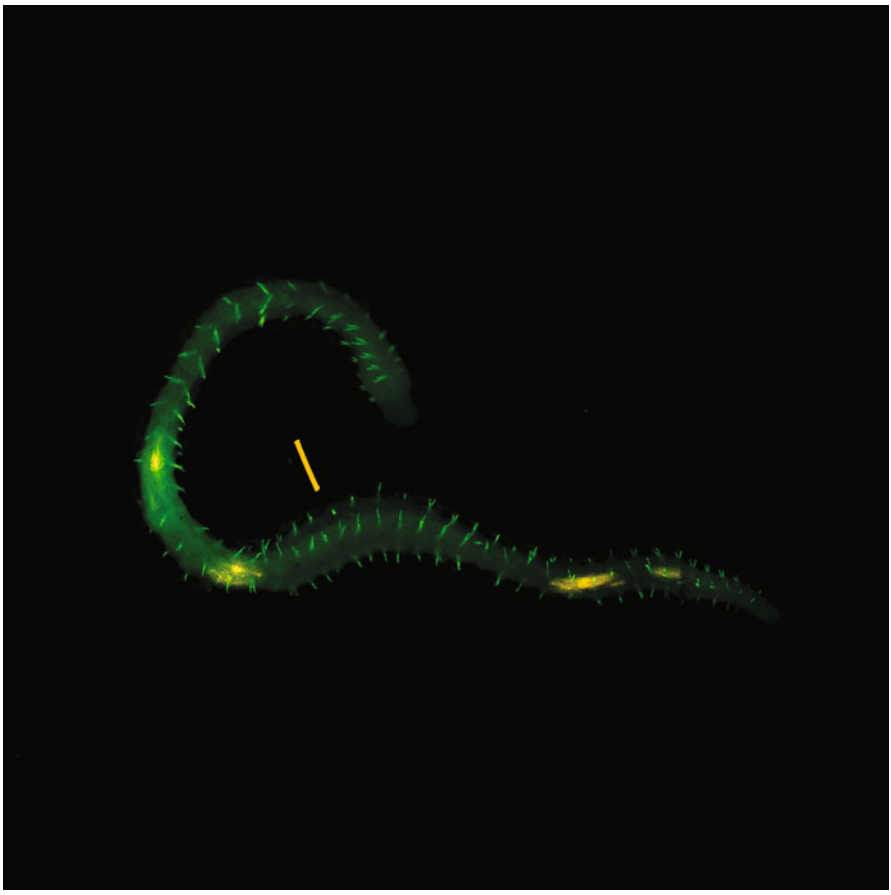

Fig. 5 Ingestion of fluorescent microplastic fibers (orange color) by an aquatic worm. The distinctly visible fiber is outside the worm (Lumbriculus variegatus). All other fluorescent particles are ingested and inside the gut. (© Christian Laforsch, University of Bayreuth)

water column to the waterbed, making them potentially more bioavailable for benthic organisms (Fig. 5).

Nevertheless, it has been shown that microplastics often remain in the digestive tract longer than natural particulate material, such as clay minerals (Wright et al. 2013a, b). Thus, organisms are confronted with this foreign substance for a longer time. With the enhanced retention period of microplastic particles within the gastrointestinal tract, the probability of bioaccumulation and biomagnification within the food web is much higher.

Trophic Transfer

Active selection of microplastic particles might occur when animals mistake plastic for food (Nelms et al. 2018). Further, microplastics are often in the size range of

particles that are eaten by indiscriminate filter feeders. Especially those microplastic particles with a neutral or a positive surface charge can further adhere to phytoplankton and can, therefore, be found on the surface of suspended seaweed, rendering also herbivorous organisms prone to microplastic ingestion. As microplastics are persistent contaminants and often retain longer in the digestive tract than naturally occurring particles, all these mechanisms can lead to bioaccumulation in organisms from different functional feeding groups. To date, more than 690 species were found to be contaminated with microplastics (Toussaint et al. 2019; Wang et al. 2019). When such contaminated prey is consumed by a predator, the latter also ingest microplastics unintentionally. Thus, even organisms that do not have a food preference in the size range of microplastics can still ingest these particles indirectly via their food. This process leads to trophic transfer of microplastics along the food web, which may result in biomagnification. As a result, even in areas contaminated with relatively low concentrations of microplastics, the continuous ingestion of microplastic-containing organisms by predators may result in high concentrations in keystone predators (Au et al. 2017).

Trophic transfer has already been demonstrated on a laboratory scale, for instance, using low-density polyethylene microspheres on a model food chain relevant to North American estuaries (Athey et al. 2020). Further, Farrell and Nelson (2013) studied the transfer from the blue mussel (*Mytilus edulis*) to the shore crab (*Carcinus maenas*), since both are prevalent species in marine environments. Their analyses confirmed that the ingestion of contaminated blue mussels determined the concentration of microplastics in the crab, which was persistent even after 21 days post-exposure. Although different laboratory studies have assessed trophic transfer in low trophic level organisms, data on trophic transfer in the wild are still scarce. However, microplastics have been found in many wild fishes' gastrointestinal tracts, making the transfer to predators likely. Nelms et al. (2018) studied a correlation between the presence of microplastic particles in the gastrointestinal tract of Atlantic mackerels (*Scomber scombrus*) and the guts of their predators, the gray seals (*Halichoerus grypus*), suggesting that trophic transfer occurs in natural environments as well.

Since it has been shown that seafood is often contaminated with microplastics, the consumption of the latter may lead to a trophic transfer up to humans, which may also have implications on human health (Smith et al. 2018).

Ingested Microplastic Particles: Possible Effects in the Gastrointestinal Tract

The effects of microplastics at an organismal level can be separated in physical and chemical effects (Campanale et al. 2020). According to the authors, physical effects are related, for instance, to the particle size, shape, and concentration of microplastics, and chemical effects are related to chemicals that are associated with microplastics.

Effects Caused by Additives

Microplastics should not be considered as chemically inert particles. Due to the plethora of additives used during their production or intended to exert specific

characteristics, the physicochemical properties of the microplastic particles may lead to chemical effects at an organismal level. Plastic additives, which enable the various material properties of plastic or incompletely converted starting materials (monomers, oligomers), can leach out in the environment or upon ingestion in the gastrointestinal tract of organisms. Among chemical additives added to the polymers in the versatile production process are plasticizers, colorants, fillers, or flame retardants, just to name a few. The plasticizer to polymer ratio strongly depends on the material's desired property but can amount up to 50%, for example, in PVC.

Carcinogenic and hormonal effects on organisms have already been proven for some of these additives. Well-known examples are bisphenol A and phthalates (Prata et al. 2020). However, the release of additives in the digestive tract of organisms is controversially discussed in the scientific community and is considered low, since a biodynamic model (Koelmans et al. 2014) has shown a negligible release of additives in the digestive tract. Nevertheless, it has been shown that rigid PVC particles (PVC without phthalate) did not affect the growth rate and the number of offspring in *Daphnia magna*, whereas flexible PVC (the phthalate DiNP was added) did show adverse effects (Schrank et al. 2019). Other types of additives are trace metals. Trace metals are used as flame retardants, stabilizers, or biocides, which have been shown to induce effects on human health. For instance, trace metals potentially induce allergic reactions; have endocrine reactivity, which enhances the probability of hormone-induced cancer; and show genotoxic effects, the formation of reactive oxygen species, and other cytotoxic effects (Campanale et al. 2020).

Effects Caused by Adsorbed Pollutants

Besides being used as additives, trace metals and other environmental pollutants like pesticides are widely discussed to adsorb from the surrounding environment to the surface of microplastic particles. In this context, once again, the physicochemical properties of the specific microplastic particle, such as hydrophobicity or surface roughness, play a critical role in the adsorption of pollutants. Once organisms ingest microplastic particles, either with additives within their polymer matrices or adsorbed pollutants from the environment, they can suffer from adverse effects. Nevertheless, the ecological relevance of this vector effect is still under discussion. The transfer of adsorbed pollutants to organisms and the resulting possible effects of these substances are considered negligible, since there is currently a consensus, resulting from laboratory and modeling studies, that the quantities of substances absorbed by this route are small compared to those absorbed directly from the water (Bakir et al. 2014; Koelmans et al. 2016).

Effects on the Gut Microbiome

Although the mere passage of plastic particles through the digestive tract may have no direct effect on organisms, it may alter the gut microbiome instead. A recent study in mice shows that the intestinal microbiome is altered by the intake of polystyrene microplastic, resulting in a disturbance of fat metabolism (Lu et al. 2018). Similar results on the murine model system have also been found by Li et al. (2020).

Microplastic exposure resulted in a significant increase in bacterial abundance and diversity in mice fed with high microplastic concentrations. Further, microplastic exposure induced intestinal dysbacteriosis and inflammation. Even in fishes, microplastic exposure leads to alterations in the gut microbiome's composition, though the mechanism is still unknown (Triebskorn et al. 2019). Furthermore, a dysbiosis (disturbance of the intestinal microbiome) after the intake of polystyrene microplastic particles in zebrafish was found (Jin et al. 2018). Whether other organisms were also affected at this level and what role the type of plastic with the corresponding physical and chemical properties plays in this is entirely unexplored. Since gut microbiomes affected by microplastics may have different effects on the immune function, further studies are required to better understand this topic and its potential threat to animal and human health (Li et al. 2020).

Microplastics: Tissue Translocation

One potential risk that has been intensively discussed but not yet sufficiently investigated and understood is the translocation of microplastic particles from the digestive tract and respiratory system into cells and tissue. It has been shown that microplastics not only pass through the intestinal tract but are also absorbed on and encapsulated within the tissue, which can lead to inflammatory responses, as shown in mussels (von Moos et al. 2012). Further microplastics were found to be translocated from the digestive tract into the mussels' circulatory system (Browne et al. 2008). In the shore crab (*C. maenas*), microplastics were detected in the hemolymph, hepatopancreas, ovaries, and gills, indicating that some particles can cross the gut epithelium (Duis and Coors 2016). The translocation of microplastics in different body compartments is not just described in invertebrates but recently also in vertebrates including zebrafish, where hyperspectral imaging was used to identify nanopolymer particles translocated from the intestine into the liver (Galloway et al. 2017a, b). Additionally, the translocation of microplastic is not exclusively found under laboratory conditions but also occurs in natural environments in fish (Barboza et al. 2020).

As mammalian model systems to study tissue translocation, mainly mice and rats or murine cell lines are used. Feeding experiments showed the translocation of micro- and nanoplastics from the gut to excretory organs like the liver and kidney (Yong et al. 2020). Particulate substances in the size range of microplastic particles, as found in environmental samples, can, therefore, potentially also be translocated into the tissue of humans, which underlines the environmental relevance of this issue.

To date, neither the corresponding interactions between the cells of the intestinal tract and the microplastic particles have been understood, nor has it been investigated whether microplastics are internalized by cells directly or which internalization mechanisms are potentially involved.

It is known that particles in the micrometer size range can, in principle, be internalized by epithelial cells and identified as potential pathogens by cells of the

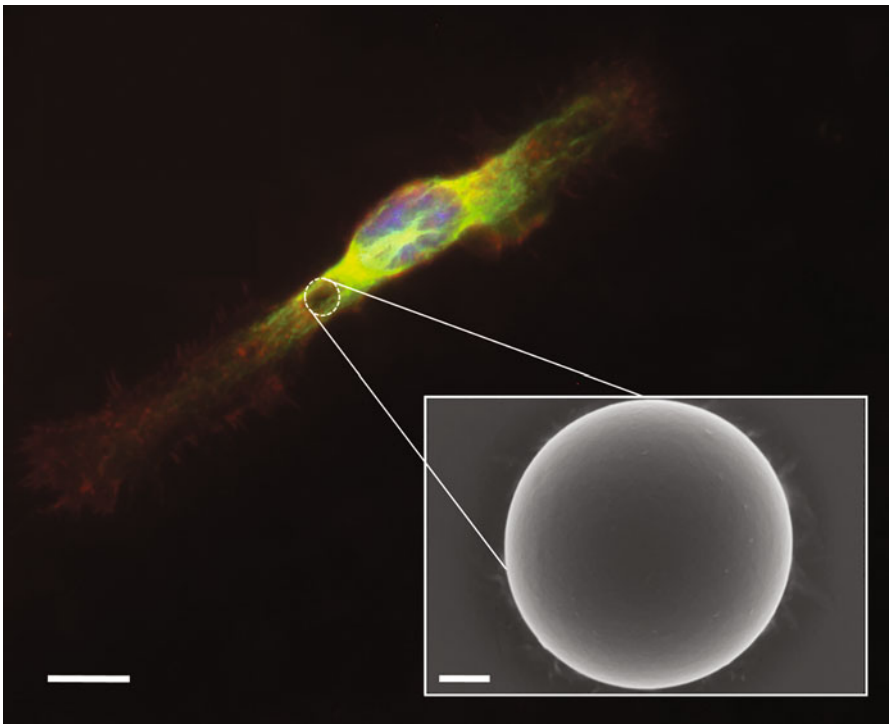

Fig. 6 Cellular internalization of a spherical microplastic particle. Fluorescently labelled J774A.1 murine macrophage cells exposed to 3 μm polystyrene microplastic particles. The cytoskeleton of the cell surrounds the microplastic particle. False-color image; blue, DAPI stain of the nucleus; red, Alexa Fluor™ phalloidin stain for filamentous actin; and green, immunolabelling of the microtubules, scale bar: 10 μm. Scanning electron microscopy image of the 3 μm spherical polystyrene microplastic particle, scale bar: 500 nm. (© Anja F. R. M. Ramsperger, University of Bayreuth)

immune system, e.g., macrophages (Fig. 6). Particles are further internalized by cells via, e.g., phagocytosis when coated with antibodies (Desjardins and Griffiths 2003).

There are mainly two possible pathways of how micro- and nanoplastic particles may translocate into tissues. Either the particles are transported paracellularly, which means in between cells through, e.g., tight junctions, or transcellularly, which means the endocytosis of particulate matter into cells directly (Wright and Kelly 2017). Endocytosis mechanisms depend on several factors. Besides receptor-mediated internalization mechanisms, which require ligand-proteins and suitable receptors, the size of the particles is an essential factor (Doherty and McMahon 2009). Smaller particles are suggested to become internalized passively, whereas larger particles in the lower micrometer size range are discussed to become internalized by cells in an energy-dependent active manner (Shang et al. 2014; Wright and Kelly 2017). However, even the particle's surface properties are responsible for tissue translocation, and therefore, the environmental coating of the particle may play a crucial role in cellular internalization (Galloway et al. 2017a, b).

The Coating of Microplastic Particles: Biofilm, Coronas, and Pathogens

Once micro- or nanoparticles enter aquatic environments, a biofilm can develop on the particles' surface. In the marine environment, the development of a biofilm has been shown on various polymer types (Oberbeckmann et al. 2015). The development of a biofilm can generally be described to occur in five successive steps. In the first step, microorganisms reversibly attach to the surface. Within the second step, microorganisms excrete extracellular polymeric substances (EPS), enabling them to stick to the surface better and, in the third step, start to proliferate within the EPS matrix. By forming 2D and 3D colonies, the biofilm grows within the fourth step. A biofilm is a highly dynamic system, as, within the fifth step, microorganisms are also able to detach from the surfaces (Renner and Weibel 2011). Next to the adhesion of microorganisms within the first step, another important factor is the adhesion of biomolecules on the particle surfaces. It has been suggested that the adhesion of biomolecules on surfaces appears within seconds (Loeb and Neihof 1975). The initial biomolecule coating is not a stable system, as biomolecules with higher binding affinities may substitute biomolecules with lower binding affinities. Over time a so-called (more or less) stable hard corona develops on the surface of the particle. On top of this hard corona, an additional corona develops, which is highly dynamic with its surrounding environment. This dynamic corona is called the soft corona and is in high exchange with its surrounding environment (Monopoli et al. 2012). The process of the development of a protein corona, especially on the surface of nanoparticles, has intensively been studied in medical research, e.g., for drug delivery using target nanoparticles.

A rather new research field is the coating with biomolecules from complex environments like marine or limnetic ecosystems. The coating with environmental biomolecules is said to be an ecocorona, referred to the similar term "protein corona." An ecocorona can consist of different kinds of biomolecules, like proteins, lipids, or carbohydrates, just to name a few (Galloway et al. 2017a). On a cellular level, it has been shown that particles coated with protein coronas interact differently with cells (Monopoli et al. 2012; Francia et al. 2019). On an organismal level, it has already been shown that the coating with an ecocorona mediates the impact of polystyrene nanoparticles to *D. magna*. Additionally, *D. magna* was less sufficient in removing particles with an ecocorona from their digestive tract compared to particles without an ecocorona. This highlights the importance of including micro- and nanoplastic particles coated with an ecocorona in future experimental attempts, as these may show severe effects that may not occur by using pristine microplastic particles (Nasser et al. 2019).

Another critical aspect that has been widely discussed in the context of environmental impact is the adherence of pathogens on the surface of plastic particles or within the biofilm matrix (Zettler et al. 2013). However, it is not clear whether or not there is a difference in pathogen load on microplastics compared to occurring natural particles coated with a biofilm (Rummel et al. 2017). This again highlights that

besides the use of pristine microplastics and plastic particles coated with an ecocorona, natural particles must also be included as a reference for the risk assessment of microplastics.

Effects on Cellular, Tissue, and Physiological Level

Once ingested, it has been shown in laboratory studies that microplastics can exert adverse histopathological effects in fish, i.e., causing damage to villi structures in the gastrointestinal tract and on the gill membranes. Further, the longer the residence time of microplastic within the organisms is, the more damage occurs to the immune system and blood parameters. In addition, alterations of metabolic profiles indicating disturbed lipid and energy metabolism were reported (Triebskorn et al. 2019). Furthermore, it has been reported that maternal exposure to polystyrene in mice led to metabolic disorders in the offspring (Luo et al. 2019). Beyond laboratory feeding experiments, Barboza et al. (2020) analyzed the microplastic ingestion and accumulation in wild fish. For fish containing microplastics in their brains, gills, and dorsal muscles, they found a significantly higher lipid peroxidation level and increased brain acetylcholinesterase enzyme activity. Some studies have also shown effects at the molecular level using different terrestrial and aquatic organisms by applying an "omics" approach (e.g., Limonta et al. 2019). Here, the authors report on alterations in the expression of immune system genes, indicating that microplastics are identified as stressors.

First attempts have been made to investigate possible negative effects on human cell lines. Similarly, when investigating effects at an organismal level, contradictory results were found. In general, the most often reported effects in human cell lines are the generation of reactive oxygen species and the increase in inflammatory responses (Yong et al. 2020), whereas, in some studies, no effects were found. This inconsistency may originate from various factors. Different cell lines may interact differently with the used particles. The sizes of the particles, as well as the concentrations used, were profoundly different and could, therefore, lead to different results. Another critical aspect in cytotoxicity studies is the choice of particles. The use of surfactants for colloidal stable particle solutions may alter the surface of the used particles. Additionally, surface-functionalized particles may behave entirely different compared to non-functionalized particles.

Microplastics: Effects on Morphology, Behavior, Population, and Life History

The ingestion and possible accumulation of micro- and nanoplastics within body compartments has been suggested to pose a risk to organisms. Depending on the sampling site, environmental concentrations can vary accordingly from few to several thousand particles/L; hence, experimental designs have to consider this. Micro- and nanoplastic particles can alter the behavior, morphology, or life history of an organism at concentrations relevant to environmental exposures. For instance, the exposure of zebrafish (*Danio rerio*) to plastic particles (100 and 1000 μg/L, 50% polystyrene +50% high-density polyethylene) resulted in

alterations of their circadian timekeeping mechanism, resulting in an increased activity during the dark and the loss of the regular diurnal pattern of activity (Limonta et al. 2019). Alterations in the phototactic behavior were shown for *D. magna*, along with increased swimming activity and reproduction after the exposure to three different polystyrene microplastic concentrations (0.125, 1.25, and 12.5 μg/mL) (De Felice et al. 2019). The authors suggested that increased swimming activity might be explained as an avoidance behavior or an attempt to eliminate the microplastic particles.

Energy depletion caused by a large number of microplastics in the digestive tract results in reduced food intake, and it can additionally lead to a significantly reduced survival rate, increased development time, and reduced fecundity, as it has been shown for copepods (*Tigriopus japonicus*) and lugworms (*Arenicola marina*) (Duis and Coors 2016). In *Daphnia magna,* microplastic exposure resulted in a reduction in the population growth rate and so in an impairment in the cladoceran's fitness, probably due to a decrease in food intake in the presence of microplastic particles (Martins and Guilhermino 2018).

Impairment in fertility and larval growth was also examined in sea urchins (*Paracentrotus lividus*). In detail, a lower fertilization rate was observed in eggs exposed to plastic particles along with larvae abnormalities and a decreased developmental time (Martínez-Gómez et al. 2017). Other studies reported similar results on plutei larvae (Messinetti et al. 2017). Adverse effects on fertility and larval development were assessed in oysters (*Crassostrea gigas*) as well. In particular, both the sperms and oocytes' numbers decreased and were deteriorated in quality compared to the control organisms. Further, the larval developmental was significantly slower (Sussarellu et al. 2016). The mentioned effects of micro- and nanoplastic exposure to organisms are not only shown for aquatic organisms. Effects from microplastic exposure have already been shown for terrestrial invertebrates, like nematodes, oligochaeta, collembola, or isopods. Studies on nematodes (*Caenorhabditis elegans*) have revealed that smaller microplastic particles impaired the survival rate, the average lifespan, and body growth. Collembolans were found to be more sensitive to microplastic exposure, showing a significant inhibition in growth and reproduction (Zhu et al. 2019).

Considering morphological alterations due to microplastic exposure, the current results are less consistent. Some suggestions have been made that the exposure to microplastic particles may also alter the morphological parameters of daphnids. For instance, it has been shown that *D. magna* shows a larger body size than the control treatment when exposed to microplastics (De Felice et al. 2019). For similar concentrations used, Eltemsah and Bøhn (2019), on the other hand, show now alterations in the body length of daphnids.

This highlights that the causes of the effects on the organisms investigated are not yet understood, primarily since exposure to the same type of plastic has led to adverse effects in some studies, but in others, no effects on the organisms investigated could be observed. Some authors suggest that the alterations on a cellular level may subsequently lead to morphological, behavioral, or life history changes. For

instance, Limonta et al. (2019) discussed that the behavioral alterations observed in zebrafish might originate from very small microplastic particles crossing the blood-brain barrier.

Microplastics in Plants: A Fairly New Research Field

A fairly new topic on micro- and nanoplastic research is the contamination of plants (Fig. 7). In general, due to the application of sewage sludge from wastewater treatment plants, which has been shown to contain a massive amount of microplastic particles and fibers (Corradini et al. 2019), or due to the application of organic fertilizers (Weithmann et al. 2018), agricultural fields can be contaminated with plastics (Piehl et al. 2018). The possible effects of micro- and nanoplastic pollution on plants can be divided into indirect and direct effects. For terrestrial plants, indirect effects may come from altered soil structure. Depending on the physicochemical parameters as well as the size and shape of the micro- and nanoplastic particles, the soil bulk density, structure, and water holding capacity may be altered (Rillig et al. 2019). This may have effects on the root growth or microbial community composition, which in turn may affect nutrient acquisition. The aquatic plant *Lemna minor* was found not to be altered

Fig. 7 Plastic and plant growth. A plastic bottle degrades on an agricultural field. (© Simona Mondellini, University of Bayreuth)

in its leaf growth rate and amount of photosynthetic pigments but negatively affected in its root growth. The authors suggest the microplastic particles being adsorbed onto the surface of the roots and mechanically blocking the root growth (Kalčíková et al. 2017). A direct effect of microplastic and nanoplastic contamination is the uptake of the particles into plant tissues. This has been suggested to be unlikely for microplastics but generally accepted for particles in the nano-size range. Recently, it has been shown that functionalized nanoplastic particles adhere to the root surface of *Arabidopsis thaliana* and reduce the root growth correlating with particle concentrations. Furthermore, the nanoparticles may even translocate into the root epidermis cells and the catheter of the xylem (Sun et al. 2020). Although the authors used functionalized particles which are unlikely to occur in nature, these findings indicate that the translocation and further accumulation of nanoplastics in plants are generally possible. Due to methodical and technical limitations in identifying nanoplastics in environmental matrices, the environmental pollution of nanoplastics is currently unknown. Nevertheless, the fact that plants used for food production may accumulate plastics within their tissues may elicit environmental and human health risks.

Microplastics Risk to Humans?

The exposure of humans to plastic particles has extensively been discussed and investigated in the last years. The ubiquitous occurrence of plastics in the environment and consumer products makes human exposure to microplastics inevitable. The most reasonable pathway is discussed to be via ingestion. Microplastic contamination of food and beverages has already been shown. For instance, microplastic particles were found in salt, sugar, processed food, and beverages like beer and drinking water and seafood (Wright and Kelly 2017). Once in the digestive system, there are several adverse effects discussed. Microplastics can potentially be adsorbed by M-cells in the intestine, penetrating the intestinal mucus (Prata et al. 2020). Moreover, the ingestion of microplastics and transfer of endocrine-disrupting chemical additives potentially could be associated with a range of chronic metabolic effects, including infertility, obesity, and cancer (Sharma and Chatterjee 2017).

A further as yet unexamined risk is the intake of microplastic particles via the air we breathe, as it has been shown that up to 16 microplastic particles can be present per m^3 of air (Vianello et al. 2019). Microplastic fibers are shown to be possibly inhaled, likely most of them undergo mucociliary clearance, but in some cases, they can persist in the lung causing obstructions and inflammation, especially in individuals with compromised clearance mechanisms. Persistence in the lungs seems to be connected to the particles' dimension. The longer fibers are the more persistent and the more likely to create obstructions or to penetrate deep in the lung. Furthermore, microplastics can determine granulomatous lesions in the lung tissues and respiratory irritation, a phenomenon mainly observed in the textile industry workers after chronic exposure (Gasperi et al. 2018).

Although the addition of plastics to cosmetics was banned in several countries (Conkle et al. 2017), the uptake via derma is generally discussed to be a possible pathway to enter human bodies, especially for nanoplastics. Nevertheless, the exposure to the associated additives such as bisphenol A or phthalates is discussed to be more alarming (Prata et al. 2020). The extent to which harmful additives such as phthalates are transferred into the human body via microplastics or directly through water, food, or contact with consumer items remains unknown, but it is known that virtually everyone has plastic-associated chemicals in their bodies (CDC 2020).

Synopsis

Overall, the mechanisms underlying the direct and indirect effects of plastics on organisms, as shown so far, are not yet understood. In general, macroplastics cause more obvious ecological effects, whereas the effects of microplastics are not so easy to elucidate. The nature of the effect probably depends mainly on the particles' physicochemical properties, the particle shape, the corresponding degradation stages, and environmental coating and concentration. Yet, the majority of the studies on biological effects were carried out with unrealistically high concentrations of microplastics, whereby the sheer quantity of foreign substances could have caused the observed effects and not the plastic particle per se.

The discrepancy that in some studies, although the same type of plastic was used, effects were found at different biological levels, but not in other studies, is due to the complexity of the issue.

As a result, there are still considerable gaps in knowledge regarding the biological effects of microplastics under realistic conditions. There is a dearth of studies that have been carried out concerning the physicochemical properties of microplastic particles and in comparison to naturally occurring particulate material since ecotoxicological studies have mostly used virgin ground plastics whose physical and chemical properties have not been characterized. Hence, more environmentally relevant studies are needed to assess the risk of microplastics for environmental and human health.

Cross-References

- ▶ Dose-Response Analysis: Identification of Threshold Levels for Chemicals
- ▶ Exposure Scenarios in Toxicology
- ▶ Green and Sustainable Chemistry as Regulatory Levers
- ▶ Importance of Physicochemical and Physical Properties for Toxicological Risk Assessment
- ▶ Nanoparticles and Their Regulation
- ▶ National and International Collaboration in Regulatory Toxicology

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Regulation of Textiles

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Britta Hilgenberg and Lutz Vossebein

Contents

Abstract

This chapter provides an overview about requirements of textiles regarding different regulation aspects. These legal requirements, among others, must be considered by, e.g., manufacturers and distributors, if textiles or clothing is to be placed on the market. At first, the regulation of harmful substances and chemicals on textiles is described. Since there are separate specifications for antimicrobials

B. Hilgenberg · L. Vossebein (✉)
Faculty of Textile and Clothing Technology, University of Applied Sciences Niederrhein, Moenchengladbach, Germany
e-mail: britta.hilgenberg@hs-niederrhein.de; lutz.vossebein@hs-niederrhein.de

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on textiles, this aspect is included, and the regulation of antimicrobial textiles is listed in particular. Furthermore, there is a brief description of regulation of personal protective equipment and medical textiles. As the legal guidelines for textile regulation are country-specific, the requirements are listed for both the European Union and the United States.

Keywords

Textile regulations · Harmful/toxic substances · Antimicrobial textiles · Personal protective equipment · Medical textiles

Introduction

Depending on the intended use, whether in the private or professional sector, customers demand high standards on clothing or protective textiles. In order to meet their specific requirements, the textile fibers used are subject to high-quality process engineering and various finishing processes to give the fabrics high-quality properties (Iheaturu et al. 2019: 23). Those properties are, for example, easy care finishing, water repellency, or flame retardancy (Choudhury 2017: 5).

As a result of these processing steps, residues of harmful substances may possibly remain on the textiles (Muthu 2020: 45–46). Since the textiles are usually worn directly on the skin or very close to the body, it is essential that the textiles do not pose any risk to the wearer.

Another important property of textiles, especially in the professional sector, is the provision of one or more protective functions. These include, for example, protection against microorganisms by antimicrobial textiles; protection of the wearer against hazards such as poor visibility, weather influences, or electrical charge by personal protective equipment (PPE), as well as the protection of patients and medical staff against infections by medical textiles.

To ensure that certain quality and safety standards are maintained by manufacturers and distributors, textiles are subject to country-specific regulations for certain aspects. This should ensure a constant high quality of the textiles, a comparability on the market, and above all a maximum security for the customer.

Regulation of Harmful Substances on Textiles

Due to impurities from the manufacturing process or addition of certain finishing substances, textiles can be contaminated with harmful chemicals. These can be, for example, organohalogen compounds (AOX), colorants, heavy metals, or formaldehyde (Muthu 2020: 45–46). In order to bring textiles on the market that are harmless to human or environmental health, the textiles must be regulated with regard to harmful chemical residues for which there are country-specific legal requirements.

Regulation of Harmful Substances on Textiles for European Market

According to European law, chemical substances have to be registered by the Regulation (EC) 1907/2006 REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) at the European Chemicals Agency (ECHA) (Regulation (EC) 1907/2006 2006). REACH was designed to improve the protection of human health and the environment from the risks posed by chemicals while increasing the competitiveness of the EU chemical industry (European Chemicals Agency [ECHA] 2020a).

At regular intervals, ECHA publishes a Restricted Substances List (RSL) with substances of very high concern (SVHC) that are either completely banned or restricted by limit values. The criteria that identify substances as SVHC are defined in Article 57 of REACH (Regulation (EC) 1907/2006 2006). These are substances with, for example, carcinogenic, mutagenic, or reprotoxic properties of category 1A or 1B or substances that are persistent, bioaccumulative, and toxic.

REACH obliges all actors in the entire supply chain of the textile and clothing industry to inform ECHA and their customers if the produced goods contain SVHC in a quantity of more than 0.1 mass percent (Haas et al. 2016: 23). Products that do not comply with the limit value regulations may no longer be placed on the market after January 11, 2020. According to Article 7(6) the notification obligation is waived if the substances have been registered for a corresponding use. Additionally, the regulation does not apply to textiles that fall within the scope of the Regulation (EU) 2016/425 on personal protective equipment (Regulation (EU) 2016/425 2016) and the Regulation (EU) 2017/745 on medical devices (Regulation (EU) 2017/745 2017).

Regulation of Harmful Substances on Textiles for US Market

According to US law, regulatory actions for specific chemicals (except food, drugs, cosmetics, and pesticides) fall under the Toxic Substances Control Act (TSCA) (15 U.S.C. §2601 *et seq*. 1976). The Environmental Protection Agency (EPA) has regulatory authority under TSCA (Environmental Protection Agency 2020e). This means that EPA evaluates potential risks for human health and the environment from new and existing chemicals and restricts the use of the chemicals if the risk is too high. The process for ensuring the safety of chemicals includes prioritization, risk evaluation, and risk management (Environmental Protection Agency 2020f). Based on information and data provided from chemical suppliers and manufacturers, EPA publishes an inventory list of the existing chemicals with possible restrictions for manufacturing or use in products (Environmental Protection Agency 2020g). Only textiles that comply with the list may be placed on the market.

Additionally, there should be mentioned briefly that in 2008, the Consumer Product Safety Improvement Act (CPSIA) came into force (15 USC §2051 *et seq.* 2008). This law makes demands on consumer goods (for children up to the age of 12) imported into the United States or produced in the United States and has a particular impact on a large number of products, e.g., apparel (Benson and Reczek 2016).

Standard 100 by Oeko-Tex

Different organizations or brands, e.g., the American Apparel & Footwear Association (AAFA), regularly publish their own RSLs in addition to the legally prescribed RSLs (American Apparel and Footwear Association 2020). In 1992, the Oeko-Tex® Service GmbH published the first RSL, even before legal requirements regarding the chemical content of textiles were available (Oeko-Tex 2020a). This RSL is updated at least annually and published in the document "Standard 100 by Oeko-Tex®" (Oeko-Tex 2020b). The aim of this globally accepted standard was the indication, by means of the label "Confidence in Textiles/Textiles Vertrauen," to the consumer that textile products are harmless to health. Textile products can only be certified according to Standard 100 by Oeko-Tex® if all components meet the required criteria, e.g., not only the fabric but also the sewing threads, prints, buttons, etc. The certificate is valid for 1 year and can be renewed afterward.

The standard considers (i) legally prohibited substances, e.g., by REACH, such as carcinogenic azo dyes; (ii) legally prescribed limit values, e.g., by CPSIA, for chemicals such as formaldehyde, plasticizers, heavy metals, and pentachlorophenol; (iii) substances that are hazardous to health but not prohibited by law, such as pesticides, allergenic dyes, and organotin compounds; and (iv) color fastness, skin-friendly pH value.

The advantage of a globally standardized and recognized certification is that a verified harmlessness of the textile is visible at first sight. This can be used, for example, by conformity assessment bodies for certification of personal protective equipment (PPE). If the products are not tested in the EU but have a valid Standard 100 by Oeko-Tex® certificate, the conformity assessment body can rely on that the requirements according to chemical content on the product are fulfilled (see section "Regulation of Personal Protective Equipment (PPE)").

Regulation of Antimicrobial Textiles

Textiles that are treated or finished with antimicrobials are considered separately for regulation, as the substances used are classified as biocides/pesticides. Again, there are different regulations for the EU and the United States that have to be satisfied regarding the applied antimicrobial substance but also the textile product itself.

Registration of Antimicrobial Textiles for European Market

When textiles are finished with antimicrobial substances in the EU, these antimicrobial substances have to be registered according to European law by the regulation REACH (Regulation (EC) 1907/2006 2006), whereas registration of antimicrobial textiles is subject to the Biocidal Products Regulation (BPR) ((EU) 528/2012 2012 and (EU) 334/2014 2014).

Registration of Chemicals by REACH

Since the European regulation REACH entered into force in 2007, substances with at least 1 ton per year manufacturing or usage quantity in Europe have to be registered at ECHA (Bundesinstitut für Risikobewertung [BfR] 2020a). Without registration chemicals may not be placed on the market or may not be further processed (Umweltbundesamt 2020). With some exceptions, which are subject to different legislations, REACH applies to all substances (chemicals) manufactured or used in the EU and also to substances in mixtures or articles (e.g., colors or clothing) (Bundesinstitut für Risikobewertung [BfR] 2020a).

To comply with the regulation, companies must identify and manage the risks associated with the substances they manufacture or place on the EU market. They must demonstrate to ECHA how the substance can be used safely, communicate the risk management measures to users, and cooperate with other companies that register the same substance (Fisk 2014). Additionally, downstream users have to provide information on the exact use of the processed substances (e.g., in antimicrobial textiles) for manufacturers or importers so that they can consider the use in their technical dossier and, if necessary, in their exposure scenarios and recommend appropriate risk reduction measures (Fisk 2014). ECHA's authorities and scientific committees assess after submission of the registration documents whether the risks of substances can be controlled. The authorities can also prohibit hazardous substances if their risks cannot be controlled (European Chemicals Agency [ECHA] 2020a).

In addition to regulations for chemical substances that are further processed in the EU, there are also regulations for the production, market provision, and marketing of antimicrobial products.

Registration of Antimicrobial Textiles According to the BPR

The Biocidal Product Regulation (EU) 528/2012 (Regulation (EU) 528/2012 2012 and Regulation (EU) 334/2014 2014) regulates the sale, supply, and use of biocidal products, except medical devices, in the EU which are defined as compounds or compositions that contain active substances that engage harmful organisms chemically or biologically.

The objective of the regulation is to harmonize the provision of biocidal products on the market and their use within the EU while ensuring a high protection level of human, animal, and environmental health (Bundesanstalt für Arbeitsschutz und Arbeitsmedizin 2020a).

The authorization of biocidal products is carried out in two steps. First, the biocidal active substance has to be approved (EU active substance test), and the biocidal active substance is included in an EU list (EU-wide positive list; published by ECHA) of approved active substances (European Chemicals Agency [ECHA] 2020b). Afterward, biocidal products containing the active substance can be approved in an authorization procedure. For this, each EU member state has a competent authority that examines the submitted information by the companies regarding efficacy, toxicology, residue, and environmental fate of the biocidal product. If the authorities have no concerns, the biocidal product will be authorized for the requested application (Bundesinstitut für Risikobewertung [BfR] 2020b).

Depending on their intended application, antimicrobial textiles do not always need to be authorized because the BPR also differentiates between treated goods and biocidal products (Bundesanstalt für Arbeitsschutz und Arbeitsmedizin 2020b). Treated goods include textiles that are finished with antimicrobial agents for their own protection against microorganisms, for example, to increase their lifetime (e.g., geotextiles or outdoor textiles). Regarding authorization, treated goods do not need to be approved. But the active substances contained must be approved for the relevant product type and intended use and have to fulfill the conditions or restrictions specified in the approval (Regulation (EU) 528/2012 2012 and (EU) Regulation (EU) 334/2014 2014). Antimicrobial textiles that claim to kill or reduce microorganisms are biocidal products and need to be authorized by the BPR.

The registration of an antimicrobial textile has a maximum duration of 10 years, but after this time an extension of the registration is possible. There is also a requirement for special labelling for information of customers or distributing companies regarding biocidal substances in antimicrobial textiles. Concerning advertisement, it is not allowed to trivialize the biocidal activity of the antimicrobial textile.

Registration of Antimicrobial Textiles for US Market

In the United States, the federal law that directs the registration, distribution, sale, and use of pesticides is the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 USC). (Environmental Protection Agency 2020a). The Environmental Protection Agency (EPA) has regulatory authority under FIFRA for pesticides which means that before a pesticide may be sold, distributed, or used in the United States, it must be registered by the EPA (Sanders 2003). If products are to be sold in different US states, they must be registered in each state in which these products are distributed. Pesticides produced by foreign manufacturers and imported for sale or distribution in the United States must comply with all requirements. This includes registering the pesticide product before starting production (Environmental Protection Agency 2020b).

FIFRA defines an antimicrobial pesticide on the one hand as any substance or mixture of substances that is intended to disinfect, sanitize, reduce, or mitigate growth of microorganisms (fungus, virus, bacteria, or other microorganisms which are not located in or on humans/animals). On the other hand, antimicrobial pesticides are supposed to protect surfaces or substances from contamination, fouling, or deterioration caused by microorganisms (Environmental Protection Agency 2020c).

Since the enactment in 1947, the main objective of FIFRA is to ensure that pesticides do not pose an unreasonable risk to human health or the environment when they are used as intended (Environmental Protection Agency 2020a). Registration requires the submission of various technical and scientific data to demonstrate that the product is safe. Depending on the intended use of the antimicrobial product or textile, there have to be, for example, proposals for product labelling, additional

toxicity tests, skin safety tests, or antimicrobial efficacy tests to enable EPA to evaluate the product for registration.

Regarding antimicrobial textiles, a distinction is made between public health and non-public health antimicrobial textiles (Environmental Protection Agency 2020d). Public health textiles claim to be effective in controlling microorganisms infectious to humans (Environmental Protection Agency 2020d). Such products need registration and efficacy test data. A sufficient effectiveness is demonstrated when the amount of test microorganisms on the textile is reduced by 99.9% in comparison to a control textile. Public health antimicrobial pesticide textiles are labeled with terms like "fights germs" or "provides antibacterial protection."

Non-public health textiles claim to control microorganisms of economic or aesthetic significance where the presence of the microorganism would not normally lead to infection or disease in humans (Environmental Protection Agency 2020d). For registration of non-public health textiles, no efficacy data are required, but they still have to be kept on file in case of data requests. The purpose of the textile has to be labeled on the product like, e.g., "control of odor-causing bacteria" or "control of bacteria which cause spoilage, deterioration or fouling."

Treated articles (specific criteria in 40 CFR 152.25 (a)) contain a pesticide only to protect the product itself and do not need registration. They make no claims about the treatment, or they claim only that it is treated to resist microbial growth (Environmental Protection Agency 2020d).

Regulation of Personal Protective Equipment (PPE)

Personal protective equipment (PPE) is any equipment intended to be used or worn by employees to protect themselves against a hazard to their safety and health. There are different types of PPE, for example, head protection, eye protection, hearing protection, respiratory protection, hand and arm protection, protective clothing, leg and foot protection, or protection against falls.

In Europe, the regulation of medical textiles is not covered in all cases by PPE Regulation but also by the Medical Device Regulation or both. For this reason, medical textiles are dealt with in a separate chapter (see section "Regulation of Medical Textiles").

Regulation of PPE for European Market

PPE for the European market must meet the requirements of the Regulation (EU) 2016/425 (Regulation (EU) 2016/425 2016) before a product is to be placed on the market. This requires that manufacturers and distributors must prove in the context of a conformity assessment procedure that the PPE actually meets the requirements for each product (European Commission 2020). Depending on the risk to which the wearer is exposed, products are divided into three different categories. The classification of PPE into categories is based on Annex I of the PPE Regulation (Regulation

(EU) 2016/425 2016). Depending on the category of the product, there is variation in the conformity assessment procedure:

- Category I (low risk): no EU-type examination is required, the EU declaration of conformity is under manufacturer's own responsibility, and an internal production control is required (Annex IV (module A)).
- Category II (every risk that is not listed under category I or category III): the conformity assessment body issues an EU-type examination certificate based on a product model as well as an EU declaration of conformity (Annex V (module B)). A regular internal production control of the manufacturer is required (Annex VI (module C)).
- Category III (high risk; PPE designed to protect against fatal risks or serious irreversible damage to health): the conformity assessment body issues an EU-type examination as well as an EU declaration of conformity (Annex V (module B)). A regular control of the product according to Annex VII, module C2, or Annex VIII, module D, is required.

Additionally, PPE must meet the so-called essential health and safety requirements. These requirements are specified in Annex II of the Regulation (EU) 2016/425. Examples for these requirements are compliance with design principles, convenience, harmlessness with regard to chemicals, presence of instructions, and information like a technical documentation (specified in Annex III). Depending on the protective function of the product, e.g., against weather, electrical charge, etc., additional requirements for the different types of PPE or for special risks have to be fulfilled. These special requirements are summarized in different standards, e.g., for high visibility (DIN EN ISO 20471 2017), electrostatic properties (DIN EN 1149-5 2018), or protection against heat and flames (DIN EN ISO 11612 2015). If the PPE product meets all requirements, this will be identified with a CE marking.

Regulation of PPE for US Market

In the United States, a PPE product for a certain industry (e.g., general industry, construction, agriculture) must meet the requirements of the Occupational Safety and Health Act (29 CFR part 1910 subpart I; part 1926 subpart E 1970) which is regulated by the Occupational Safety and Health Administration (OSHA). OSHA sets and enforces standards and additionally provides training, outreach, education, and assistance for PPE. Furthermore, depending on the security level, the product is classified, and performance tests have to be carried out at a nationally recognized testing laboratory.

Separate standards have also been defined for diverse application areas. These include PPE for the fire service (National Fire Protection Association NFPA Standard) or the US army (Army Regulation AR 385-10 2017). Since the description of the individual standards is too complex for this chapter, they will not be discussed further.

Regulation of Medical Textiles

Medical textiles are designed to protect the patient as well as medical personnel against, e.g., injuries caused by laser beams or the transmission of infectious agents. A distinction is made, for example, between radiation protection clothing, mouth-nose protection, and surgical clothing.

Regulation of Medical Textiles for European Market

In contrast to the United States, in Europe the classification of a textile as a medical device or as PPE depends on the intended purpose of the product. If the product is intended to protect a patient, the product is a medical device. If the product is supposed to protect the user against one or more risks to his health or safety, the product must be classified as PPE. A simultaneous classification as a medical device and PPE is possible if a product serves both purposes. In this case the requirements of the Medical Device Regulation (EU) 2017/745 and the PPE Regulation (EU) 2016/425 2016 have to be fulfilled.

In order to obtain a marketing authorization for medical devices in Europe, proof must be provided that essential performance and safety requirements meet the Medical Device Regulation (EU) 2017/745 (Regulation (mEU) 2017/745 2017). The conformity assessment procedure of medical devices which is carried out of a conformity assessment body is class dependent (class I, class II a, class II b, or class III) and in some points similar to the procedure for PPE, e.g., provision of a technical documentation, testing, and quality assurance. The fact that the basic requirements for a medical device are fulfilled is also documented by the CE marking.

Regulation of Medical Textiles for US Market

In the United States, the OSHA requires medical personnel to wear PPE for their protection against infections (Kilinc 2016). Depending on the intended use, this PPE may be certified as a medical device (FDA 2020a). Medical devices are regulated by the Food and Drug Administration (FDA) in order to meet the Federal Food, Drug, and Cosmetic Act (FD&C Act)(21 USC ch. 9). FDA's Center for Devices and Radiological Health (CDRH) is responsible for regulating firms who manufacture, repackage, re-label, and/or import medical devices sold in the United States. Depending on the intended use of the medical device, the products are classified (FDA 2020a):

- Class I (low risk): either no premarket approval or a 510(k) process (21 CFR Part 807 Subpart E) is required; general controls have to be conducted.
- Class II (moderate risk): a 510(k) process or a premarket approval process (PMA) (21 CFR Part 814) is required for approval; general or special controls have to be conducted.

- Class III (high risk): devices have to be approved by the PMA process; general controls or special controls have to be conducted.

For example, due to its barrier performance (ANSI/AAMI PB70:2012) against liquid and microorganisms, a medical gown is subdivided into three types of gowns (FDA 2020b) with different classification as medical device: non-surgical gowns (class I), surgical gowns (class II), and surgical isolation gowns (class III) (21 CFR 878.4040).

Besides a premarket approval, there is a need for, e.g., performance tests, special labelling of the product, and proof and compliance of quality system regulations or medical device reporting (FDA 2020b).

Conclusion

This chapter shows that a regulation of textiles is very comprehensive and can vary widely depending on the intendant use of the textile. In Europe as well as in the United States, strict and complex regulations are established, and compliance with them is monitored. However, these regulations are essential to ensure that certain quality and safety standards of the products are maintained and that users can rely on the highest possible level of safety and protection.

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► Defence of Biocidal Active Substance Dossiers in the Context of Regulatory Toxicology
► Registration and Approval in Regulatory Toxicology

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Regulation and Mitigation of Greenhouse Gases

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Stefan Lechtenböhmer and Wolfgang Obergassel

Contents

S. Lechtenböhmer (✉)
Wuppertal Institute for Climate, Environment, Energy, Wuppertal, Germany

Lund University, Lund, Sweden
e-mail: stefan.lechtenboehmer@wupperinst.org

W. Obergassel
Wuppertal Institute for Climate, Environment, Energy, Wuppertal, Germany
e-mail: wolfgang.obergassel@wupperinst.org

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Abstract

While most greenhouse gases are not toxic and no pollutants, their increasing concentration in the atmosphere is enhancing the greenhouse effect and causing climate change. Climate change however is a massive threat to all global life-sustaining systems, and it will among other highly damaging consequences probably also create significant health problems. This chapter first explains the natural science basics of the greenhouse effect and its link to the global commercial energy system. Second, it outlines the challenges connected to changing the global energy systems and what changes would be needed to prevent dangerous global warming. Third, the global governance system is presented which has been established at the United Nations since 1992 to address climate change. Finally, the chapter discusses what societal changes would be necessary and enable the "great transition" to a just and sustainable global society, how the current Coronavirus pandemic could be a start to such a transition, and how the recently announced European Green Deal might be a prototype policy on this way.

Keywords

Carbon dioxide · Climate change · Greenhouse gases · UN Framework Convention on Climate Change · UNFCCC · Kyoto Protocol · Paris Agreement · Sustainable Development Goals · SDGs · Great transition · Climate neutrality · Energy system

Abbreviations

BAP	Bali Action Plan
BC	Before Christ
BECCS	Bio energy and CCS
CA	Cancún Agreements
CCS	Carbon capture and storage
CDM	Clean Development Mechanism
CFC-11	Chlorofluorocarbon
CH_4	Methane
CHP	Combined heat an power generation
CMP	Conference of the Parties serving as Meeting of the Parties to the Kyoto Protocol
CO_2	Carbon dioxide
COP	Conference of the Parties
EJ	Exa Joule (10^{18} Joule)
GCF	Green Climate Fund
GHG	Greenhouse gases
GMST	Global mean surface temperatures
GST	Global Stocktakes
Gt	Giga tons (10^9 tons)
GWP	Global warming potential

H_2O	Water vapor
HFC-134a	Hydrofluorocarbon
IEA	International Energy Agency
IPCC	Intergovernmental Panel on Climate Change
IRENA	International Renewable Energy Agency
JI	Joint Implementation
KP	Kyoto Protocol
LED	Low energy demand
MDGs	Millennium Development Goals
N_2O	Nitrous oxide
NAMA	Nationally appropriate mitigation action
NDC	Nationally Determined Contribution
NF_3	Nitrogen trifluoride
NGO	Non-governmental organization
O_3	Ozone
PA	Paris Agreement
ppm	Parts per million
RES	Renewable energy sources
SDG	Sustainable Development Goals
SF_6	Sulfur hexafluoride
SPM	Summary for policy makers
UN	United Nations
UNCED	The United Nations Conference on Environment and Development
UNEP	United Nations Environment Programme
UNFCCC	UN Framework Convention on Climate Change
WBGU	German Advisory Council on Global Change
WHO	World Health Organization
WMO	World Meteorological Organization

Introduction

The so-called greenhouse gases have no adverse health effects at the concentrations that prevail in the atmosphere. The two most important, water vapor and carbon dioxide, are natural components in the atmosphere. However, due to the massive burning of fossil energy carriers, such as coal, oil, and natural gas, the human economy is increasing the atmospheric content of carbon dioxide and other greenhouse gases in the atmosphere. For carbon dioxide, the concentration has increased by almost 50% above the levels that prevailed before the beginning of the Industrial Revolution in the mid-nineteenth century. Thereby, the beneficial balance of the greenhouse effect is altered, and global mean temperatures are bound to increase with very significant consequences for virtually all life on Earth.

Although the effect was detected and already described by Swedish scientist Svante Arrhenius in the late nineteenth century, it took the global community almost a century before starting to act. In 1992 at the Earth Summit in Rio de Janeiro, the

UN Framework Convention on Climate Change (UNFCCC) laid the foundations for a global governance system to tackle climate change. However, as basically all economic activities are linked to the use of energy from fossil fuels, reducing their use is a huge challenge which affects all areas of daily life. In spite of their political aims, so far the global community has not been able to stop the still growing trend of greenhouse gas emissions. Therefore, significant action becomes ever more urgent if catastrophic consequences from climate change are to be prevented.

Today the world is at crossroads regarding climate change. Virtually in these days, the world is reaching the point after which it will be impossible to prevent global temperatures to rise beyond the level of dangerous interference with the climate system. At the same time, due to the global Coronavirus crisis, the world has for the first time in years seen falling greenhouse gas emissions in 2020. If nations around the globe are able to use the current crisis and the immense expenditures that are being undertaken to buffer its consequences on the global economy as a catalyst to change the trajectories of the energy system toward a more sustainable route, the world may currently see its probably last chance to escape some of the worst potential consequences of climate change. If, however, a "back-to-normal fossil energy use" will dominate the coming months and years, the world will probably not be able to bend the curve fast enough later. This will mean that the world will set itself inevitably on a highly dangerous climate trajectory.

In the following, first the basics of the greenhouse effect and its link to the global commercial energy system are explained. The second section sketches the challenge of changing global energy systems and what changes would be needed to prevent dangerous global warming. The global governance system established since 1992 is then described in the third section. Finally, the fourth section discusses what societal changes would be necessary and enable the "great transition" to a just and sustainable global society, how the current crisis could be a start to such a transition, and how the recently announced European Green Deal might be a prototype policy on this way.

What this chapter does not cover is the vast field of adaptation of human societies and natural ecosystems to the impacts of climate change that can no longer be avoided.

Global Warming and the Greenhouse Effect

The fact that the Earth is habitable depends on its unique atmosphere which provides air to breathe, water, and mild temperatures. The moderate temperatures on the surface of the globe are mainly resulting from the fact that the atmosphere traps large parts of the sunlight reaching the planet and converts it to heat, which is then distributed all over the planet's surface via mass movements of air and water.

This trapping function can be compared to a greenhouse, which also traps sunlight to keep its inside warmer than its surroundings. Its basic physics is explained briefly in sections "Brief Explanation of the Greenhouse Effect" and

"Overview of Greenhouse Gases and Their Global Warming Potential." Unfortunately, the amount of gases in our atmosphere (the atmospheric concentration of greenhouse gases) that create this beneficial greenhouse effect is rising. This leads to a higher radiative forcing, i.e., higher amounts of solar radiation are trapped and the greenhouse – our habitat on the Earth's surface – is warming. Section "Global Warming Caused by the Greenhouse Effect" briefly sketches global mean temperature fluctuations since the last Ice Age and what change is expected for the coming decades. Section "Climate Damages and Tipping Points: Why Is It Necessary to Limit Global Warming?" then explains why the rapid changes in global mean surface temperatures that we are witnessing today and expecting for the decades to come are so dangerous for nature and humankind.

Brief Explanation of the Greenhouse Effect

For understanding climate change, it is important to distinguish between the terms "climate" and "weather." The term "weather" refers to the short-term condition in terms of temperature, precipitation, wind, etc. at a specific location at a specific time. The term "climate," on the other hand, refers to the long-term average of the weather over a certain period of time. Normally, these variables are averaged over a period of 30 years. Climate change therefore means a change in the long-term average or the variability of the variables (IPCC 2018a). For decades, in particular, the average global air temperature has been rising steadily despite annual fluctuations. It is currently around 1 °C above the average before the start of industrialization (IPCC 2018c).

Box: The Intergovernmental Panel on Climate Change (IPCC)

While the basic mechanisms of the greenhouse effect and climate change had been described correctly already in the nineteenth century, it was only in the 1980s that there was an increasing realization that climate change was indeed occurring and was going to cause severe impacts. As one reaction, in November 1988, the United Nations Environment Programme (UNEP) and the World Meteorological Organization (WMO) established the Intergovernmental Panel on Climate Change (IPCC). Nowadays, nearly all countries of the world are members of the IPCC (IPCC Website 2020).

Its task is to investigate the risks and consequences of climate change and to present strategies and measures for avoiding dangerous climate change and for adapting to it. The IPCC does not conduct any research of its own but compiles the results of existing research in so-called Assessment Reports and Special Reports. Its latest (fifth) Assessment Report was published in 2013/2014, but scientists are currently working on the sixth Assessment Report due in 2021 or 2022.

(continued)

Each Assessment Report consists of three volumes, each of which is prepared by a specific working group. Working group 1 addresses the scientific basis of climate change, i.e., the details behind the facts outlined in section "Global Warming and the Greenhouse Effect." Working group 2 addresses the vulnerability of socioeconomic and natural systems to climate change and its impacts as well as ways in which people can adapt to global warming, topics not covered in this chapter. Working group 3 discusses political and technological measures to mitigate climate change, i.e., aspects touched upon here in section "Current and Future Emissions of Greenhouse Gases."

The Assessment Reports undergo a multi-stage peer review in three consecutive rounds, including reviews by governments and outside experts. In addition, independent review editors make sure that the revisions take due account of all the comments. Each report contains a summary for policy-makers. These summaries are discussed and adopted line by line by governments in a plenary session of the IPCC. At the end of the procedure, the IPCC plenary approves the entire report, including the summary for policy-makers (IPCC 2013a). By this procedure, governments officially recognize the validity of the scientific statements made in the IPCC reports.

Most of the material presented in this chapter draws on several reports by the IPCC.

Climate change can therefore not be "proved" or "disproved" by a particularly hot or cold season or year. Only the long-term trend is meaningful. The change in the global average temperature should also not be measured by fluctuations in daily temperature. Seemingly small changes in average temperature quickly have considerable consequences. The difference in global average temperature between today and the last glacial maximum at around 20,000 BC, when vast ice sheets covered much of North America, Northern Europe, and Asia, is only 5 °C (Otto-Bliesner et al. 2006). Even the warming of about 1 °C that has occurred so far already has clear consequences for food and water supplies (see further below).

Central to climate change is the so-called greenhouse effect. In a greenhouse, sunlight passes through the glass roof and walls largely unhindered and heats the greenhouse. The heat cannot escape through the roof and walls. A similar effect occurs in the atmosphere due to the so-called greenhouse gases (GHG). From the sun, high-energy short-wave radiation falls to the Earth and penetrates the atmosphere to the Earth's surface largely unhindered. As the Earth's surface is absorbing the short-wave radiation, it warms up and emits the heat in the form of long-wave radiation. This radiation is to a large extent absorbed by the GHGs in the atmosphere and then radiated again in all directions (see Fig. 1). The GHGs thus retain a part of the heat energy on the Earth's surface that would otherwise be radiated into space. It is the natural greenhouse effect that made life on Earth possible in its present form in

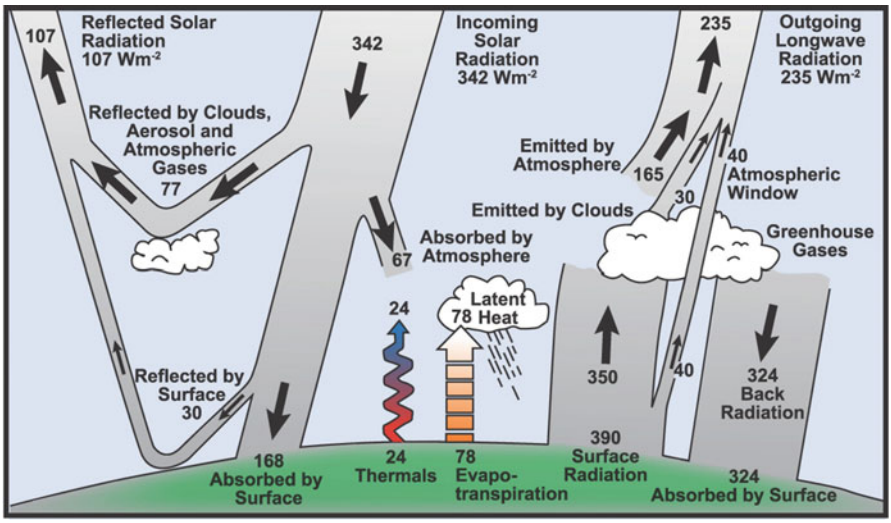


Fig. 1 Estimate of the Earth's annual and global mean energy balance. Over the long term, the amount of incoming solar radiation absorbed by the Earth and atmosphere is balanced by the Earth and atmosphere releasing the same amount of outgoing long-wave radiation. About half of the incoming solar radiation is absorbed by the Earth's surface. This energy is transferred to the atmosphere by warming the air in contact with the surface (thermals), by evapotranspiration, and by long-wave radiation that is absorbed by clouds and greenhouse gases. The atmosphere in turn radiates long-wave energy back to Earth as well as out to space. (Source: Le Treut et al. 2007, FAQ 1.1, Figure 1, with kind permission of IPCC Secretariat, Web Information and Publications)

the first place: it causes the Earth's surface to warm by 33 °C, raising the average global temperature to about 14 °C. Without the natural greenhouse effect, it would be about −18 °C (IPCC 2007).

Overview of Greenhouse Gases and Their Global Warming Potential

Various GHGs occur naturally, including water vapor (H_2O), carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and ozone (O_3) (IPCC 2018a). Through various industrial and agricultural activities, humans are increasing the concentration of greenhouse gases in the atmosphere. CO_2 is the main contributor to the anthropogenic greenhouse effect. It is released by the combustion of fossil fuels – lignite and hard coal, crude oil, and natural gas – and the progressive clearing of forests. Since the beginning of industrialization, humanity has increased the atmospheric CO_2 concentration from 280 ppm (parts per million, i.e., parts of carbon dioxide per million parts of the atmosphere) to about 410 ppm. This value exceeds by far the natural range of atmospheric CO_2 concentration of the last 800,000 years, which could be determined by ice drilling (Lindsey 2020).

Other GHGs emitted by humanity are (IPCC 2007):

- Methane (CH_4), which is emitted during the production and transport of coal, natural gas, and oil. Livestock rearing, rice cultivation, and other agricultural practices also cause methane emissions, as well as the decay of organic waste in solid waste landfills.
- Nitrous oxide (N_2O), which is emitted by certain agricultural and industrial activities, combustion of fossil fuels and solid waste, as well as during treatment of wastewater.
- Fluorinated gases do not occur naturally and are entirely human-made: Hydrofluorocarbons, perfluorocarbons, sulfur hexafluoride, and nitrogen trifluoride are synthetic gases that are emitted from various industrial processes.

All these gases have differing impacts on the climate system, depending on their behavior in the atmosphere and their atmospheric lifetime. GHGs are compared according to their global warming potential (GWP), which is a measure how much heat a GHG captures in the atmosphere during a specific timeframe. CO_2 is defined as the baseline with a GWP of one over all time periods.

Table 1 shows the GWP of the main categories of anthropogenic GHGs.

One of the reasons why it is so difficult to solve the climate problem is that almost all economic activities emit greenhouse gases. The generation of electricity and heat accounts for the largest share of these emissions, accounting for a quarter. Agriculture and forestry are only just behind with 24%. Emissions from these sectors are mainly due to the clearing of forests in southern countries and high emissions from livestock farming and rice cultivation. Other high emissions come from industry with 21% and transport with 14% (IPCC 2013b).

Table 1 Atmospheric lifetime and GWP relative to CO_2 at different time horizons for various greenhouse gases

Gas name	Lifetime (years)	Global warming potential (GWP) for a given time horizon without feedbacks	
		20 years	100 years
Carbon dioxide (CO_2)	N.a.[a]	1	1
Methane (CH_4)	12	84	28
Nitrous oxide (N_2O)	121	264	265
HFC-134a (hydrofluorocarbon)	13.4	3710	1300
CFC-11 (chlorofluorocarbon)	45.0	6900	4660
Sulfur hexafluoride (SF_6)	3200	17,500	23,500
Nitrogen trifluoride (NF_3)	500	12,800	16,100

Source: Own compilation based on IPCC 2013b

[a]For CO_2, it is difficult to give a single estimate of its lifetime, as a part of it is rather quickly absorbed, e.g., by oceans, but other parts remain in the atmosphere for a very long time (https://www.yaleclimateconnections.org/2010/12/common-climate-misconceptions-atmospheric-carbon-dioxide/)

Global Warming Caused by the Greenhouse Effect

History

The relation between the atmospheric concentration of greenhouse gases, the radiative forcing (i.e., the increased retention of long-wave heat radiation within the atmosphere), and global mean surface temperatures has been well established by science (see section "Overview of Greenhouse Gases and Their Global Warming Potential"). This has been shown by physical modelling as well as observations of past global trends. While the physical basics, i.e., the fact that greenhouse gases trap heat radiation, are well known, it is difficult to measure the effects on the mean temperature of such a big and complex system as the Earth as a whole. Latest research therefore has mainly concentrated on better understanding how the Earth's mean surface temperature has changed in different places over land and over sea and in different layers of the atmosphere and over time, as well as how the energy stores provided by the oceans, the glaciers, and big ice sheets have changed. This has been done by "diverse measurement groups in multiple countries using different technologies, investigating various climate-relevant types of data, uncertainties and processes" (IPCC 2007, p. 129).

Figure 2 shows that global average CO_2 concentrations have been slightly increasing over the last 10,000 years since the last ice age as proven by ice core measurements. However, since the twentieth century, their levels have been increasing at a speed that has been unprecedented over the 10,000 years before (Holocene epoch). Global mean temperatures have shown a largely similar trend with a strong increase since the beginning of the industrial revolution around 1850 (see Fig. 3). Today, average temperatures have reached levels around 1 °C above preindustrial levels and are rising with seemingly increasing speed. The figure shows that by now global mean surface temperatures have left the range of temperatures seen in the

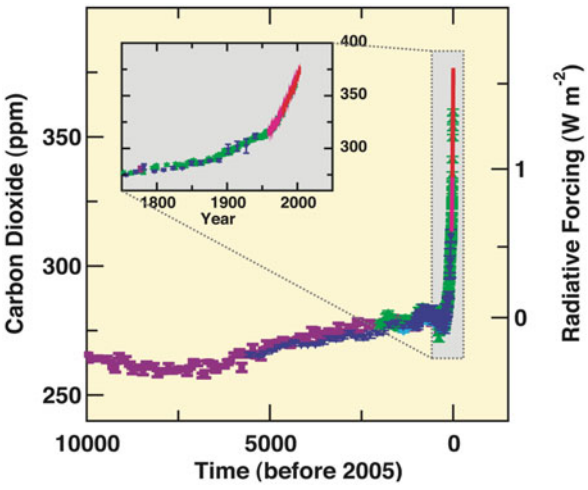


Fig. 2 Atmospheric concentrations and corresponding radiative forcing of carbon dioxide over the last 10,000 years. Measurements are shown from ice cores (symbols with different colors for different studies) and atmospheric samples (red lines). The corresponding radiative forcings are shown on the right-hand axe of the large panel. (Source: IPCC 2007, Figure SPM.1 with kind permission of IPCC Secretariat, Web Information and Publications)

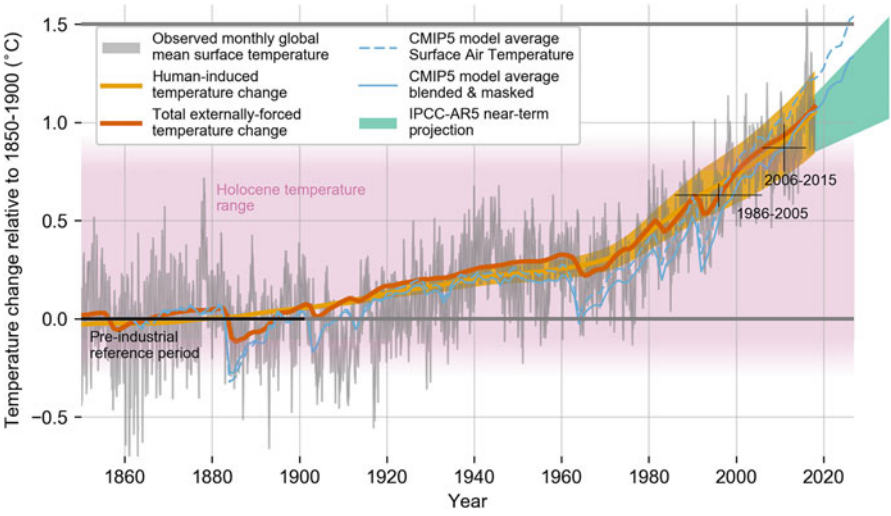


Fig. 3 Evolution of global mean surface temperatures (GMST) over the period of instrumental observations. Gray shaded line shows monthly mean global mean surface temperatures from different datasets, expressed as departures from 1850 to 1900, with varying gray line thickness indicating inter-dataset range. All observational datasets shown represent GMST as a weighted average of near surface air temperature over land and sea surface temperature over oceans. Human-induced (yellow) and total (human- and naturally forced, orange) contributions to these GMST changes are shown calculated following Otto et al. (2015) and Haustein et al. (2017). Fractional uncertainty in the level of human-induced warming in 2017 is set equal to ±20% based on multiple lines of evidence. Thin blue lines show the modelled global mean surface air temperature (dashed) and blended surface air and sea surface temperature accounting for observational coverage (solid) from the CMIP5 historical ensemble average extended with RCP8.5 forcing (Cowtan et al. 2015; Richardson et al. 2018). The pink shading indicates a range for temperature fluctuations over the Holocene (Marcott et al. 2013). Light green plume shows the AR5 prediction for average GMST over 2016–2035 (Kirtman et al. 2013). (Source: Allen et al. 2018, Figure 1.2 with kind permission of IPCC Secretariat, Web Information and Publications)

Holocene (i.e., since the Ice Age) and are well on track to reach 1.5 °C warming soon.

Future

How global mean surface temperatures will develop in the coming years depends on three main factors. The first factor are the greenhouse gases that have already been emitted. A significant share of the main anthropogenic greenhouse gas CO_2 remains in the atmosphere for thousands of years. Therefore, it will contribute to warming for a very long time. Other greenhouse gases such as CH_4, however, stay in the atmosphere for shorter periods, so the effects of past emissions will fade sooner. The second factor increasing warming are the future emissions of greenhouse gases, as they further add to the already increased warming potential. The third factor are feedback effects by the Earth system. These are so-called “flywheel-effects” and “tipping points” that are to be expected due to temperature rises already seen. For

example, the oceans have stored a significant share of additional warming over the last decades, as they warm up very slowly. Now this might change and they may not store that much of the additional warming any more, and they will prevent temperatures from declining for long. Other effects are the melting ice shields and declining snow covers in high latitudes. With less white ice and snow on the Earth's surface, more solar radiation is absorbed by the now darker surfaces of land and sea which again accelerates warming.

Figure 4 shows how future global mean surface temperatures could develop under a set of assumptions on future GHG emissions. Due to many uncertainties in knowledge and particularly feedbacks by the Earth system, these projections have wide ranges of uncertainty as depicted for the case of no future GHG and aerosol emissions. The figure shows (in yellow) that for the hypothetical case that the world would basically stop emitting anything now, temperatures would probably still rise

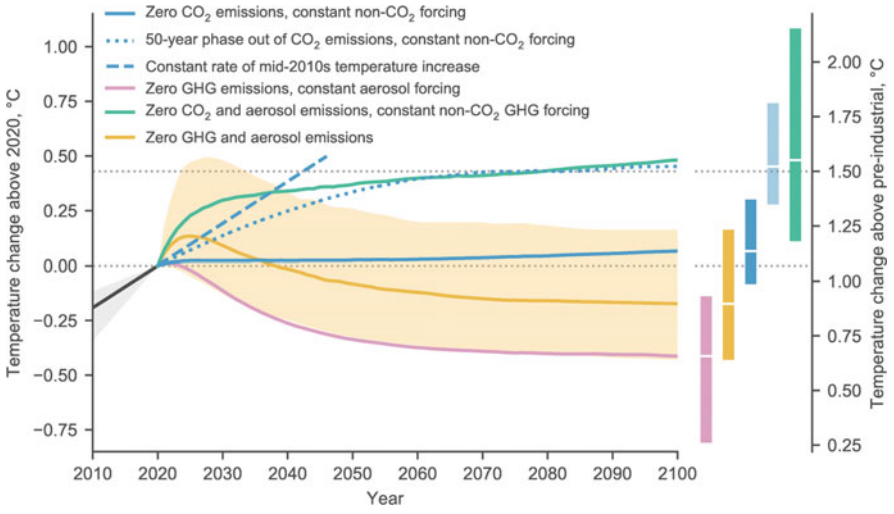


Fig. 4 Warming commitment from past emissions of greenhouse gases and aerosols. Gobal mean surface temperature change for scenarios with different combinations of greenhouse gas and aerosol precursor emissions reduced to zero in 2020. Variables were calculated using a simple climate-carbon cycle model (Millar et al. 2017) with a simple representation of atmospheric chemistry (Smith et al. 2018). The bars on the right-hand side indicate the median warming in 2100 and 5–95% uncertainty ranges (also indicated by the plume around the yellow line), taking into account one estimate of uncertainty in climate response, effective radiative forcing and carbon cycle sensitivity, and constraining simple model parameters with response ranges from AR5 combined with historical climate observations (Smith et al. 2018). Temperatures continue to increase slightly after elimination of CO_2 emissions (blue line) in response to constant non-CO_2 forcing. The dashed blue line extrapolates one estimate of the current rate of warming, while dotted blue lines show a case where CO_2 emissions are reduced linearly to zero assuming constant non-CO_2 forcing after 2020. Under these highly idealized assumptions, the time to stabilize temperatures at 1.5 °C is approximately double the time remaining to reach 1.5 °C at the current warming rate. (Source: Allen et al. 2018, Figure 1.5, with kind permission of IPCC Secretariat, Web Information and Publications)

during the 2020s but decline after 2030 and reach a level of about 0.8 °C above preindustrial levels by the end of the century (right axis). There would, however, be a small chance that temperatures by 2100 would still be higher than today. The dotted blue line shows what would happen if CO_2 emissions were reduced to zero globally over the next 50 years. In that case, global temperatures would almost stabilize with a 50% chance at 1.5 °C above preindustrial levels. Further reductions in other GHG emissions could further reduce temperature levels but only slightly. However, GHG mitigation typically goes hand in hand with reduction of air pollutants which reduces atmospheric contents of, e.g., sulfur dioxide. This is very good for health and environment, but current levels of these aerosols (or simply spoken air pollution) have a cooling effect on the global atmosphere as they reflect some of the incoming solar radiation back to space. However, this effect will probably decline as emissions are reduced.

These rather simple modelling exercises demonstrate what it would take to stabilize global warming to something around 1.5 °C above preindustrial levels, as agreed by international climate policy (see following sections): CO_2 emissions need to be phased out over the next 50 years, and other GHG emissions also need to be reduced quickly, if the chance of reaching the target shall be above 50%.

Climate Damages and Tipping Points: Why Is It Necessary to Limit Global Warming?

Climate change is already having noticeable and overall negative effects on global crop yields and the availability of drinking water. This development will intensify in the future. Due to rising temperatures, many types of food will produce lower yields in the areas where they were previously grown; some will no longer be able to be grown there at all. In addition, climate change will lead to a shift in precipitation patterns: in many regions where there is already little precipitation today, even less will fall in the future. As a result, the yields of rain-irrigated agriculture will fall sharply in some regions. The changing precipitation patterns will also significantly affect the availability of drinking water. Numerous rivers that are central to the drinking water supply are currently supplied with water from melting snow and ice in the summer, especially in the dry season. As the glaciers melt, these natural water reservoirs will decline or disappear, and water supply of the rivers will increasingly fluctuate with much higher chances of draught in the dry seasons. In addition, rising sea levels in coastal areas and on smaller islands lead to salinization of groundwater and soil. Sea-level rise also threatens the very existence of many small island states, many of which are only barely higher than the current sea level (IPCC 2014a).

Furthermore, there is a high likelihood of sudden, nonlinear, and irreversible changes if certain “tipping points” are crossed. While the precise levels of climate change that would trigger a tipping point are uncertain, if one was triggered, the impacts would be highly negative. Potential tipping points that have been identified by climate science include (Lenton et al. 2008, 2019):

- The Atlantic Meridional Overturning Circulation, which brings warm water from the Gulf of Mexico to Europe and transports cool water southward, could be shut down by the influx of freshwater from the Polar ice melt.
- The Greenland ice sheet and the West Antarctic ice sheet could disintegrate, each of which would raise sea levels by several meters.
- The Amazon rainforest as well as boreal forests in Northern latitudes could die back as a result of weather changes, increasing forest fires and insect disturbances, which would release the carbon currently stored in the forest biomass into the atmosphere.
- The permafrost in Northern latitudes could melt, which would lead to the release of methane hydrates (i.e., strong greenhouse gas emissions of CH_4) currently locked into the permafrost.
- Coral reefs, which are crucial ecosystems and support fishing and tourism in many countries, may die off irreversibly.
- The Indian monsoon could shift, endangering food supplies in the world's most populated region.

Overall, climate changes will be far more pronounced in the tropical and subtropical climate zones – where the majority of the so-called developing countries are located – than in the temperate climate zones, which is predominantly home to industrialized countries. It is therefore precisely those people who have contributed least to the effects of global warming who will suffer most. Moreover, many of the people living in these regions lack the physical, social, economic, and technological resources to adapt to the forthcoming climate changes. People and population groups who are disadvantaged by society on the basis of their gender, age, illness, or membership of a minority and who are already suffering from hunger and poverty are therefore particularly vulnerable. These are above all the rural population, especially small farming families, landless farmers and day laborers, and nomads (IPCC 2014a).

For a long time, climate policy had operated under the assumption that such impacts would be manageable if global warming was stabilized below 2 °C. However, new scientific findings over the last decade have increasingly led to the realization that severe impacts must be expected already at 2 °C and that some tipping points may already be crossed at this level of warming. In 2018, a special report by the Intergovernmental Panel on Climate Change (IPCC) concluded that the differential of risk is indeed substantial. For example, if warming was stabilized at 1.5 °C, a part of the global coral reefs might be saved, while probably nearly all coral reefs will die off if warming is stabilized only at 2 °C (IPCC 2018c).

Current and Future Emissions of Greenhouse Gases

The discussion above makes it clear how dangerous the emission of greenhouse gases is. It is therefore extremely relevant to reflect on future trajectories of greenhouse gas emissions, which are strongly linked to the core economic activities

ranging from housing, transport, industrial activities, and waste treatment to agriculture and land use. The following section gives some details about the main activities causing the emission of greenhouse gases and their relative shares. It also shows how these emissions would develop if the current level of action to reduce GHG emissions remained unchanged and how they would need to develop if the world was to achieve its targets on mitigating dangerous climate change. Section "What Would It Take to Stay Within the Limits of the Climate System?" provides more detail on what it would mean to reduce emissions so far that the 1.5 °C target would be achievable. Finally, section "The Economics of GHG Emissions and Their Mitigation" discusses what it would cost to change the trajectory of the global energy system and which sectors would be losers or winners.

Scenarios of Future GHG Emissions

Anthropogenic emissions of CO_2 and other greenhouse gases are mainly stemming from our energy use and energy systems plus from agriculture as well as burning and destruction of forests and moors. With growing population and wealth, energy needs as well as GHG emissions have steadily grown over the last decades – with only small interruptions due to the financial crisis from 2008 to 2010 or the oil price crises of the 1970s – and are expected to rapidly increase in the future if no countermeasures are taken.

As these are the underlying systems and energetic drivers of our whole economy, or as it is often put our "industrial metabolism" as a society, changes are difficult and will be felt everywhere. In other terms, the energy use of our homes, as well as of the factories producing the goods we consume and of shops, hospitals, and other service sector installations emits CO_2 and other GHGs. Cars, trucks, trains, ships, and airplanes add to this. Agricultural and particularly agro-industrial production are further important emitters of greenhouse gases with the production of beef being particularly relevant (see for the 2010 absolute emissions of these sectors the dashed lines in Fig. 5 below). This list alone shows that avoiding GHG emissions needs many changes in many places which affect all parts of our daily lives as well as all parts of our economies – not to speak of the underlying effects for income, power, and wealth.

As already mentioned, the global energy system is rapidly developing with increasing wealth in the industrialized countries of the Global North, as well as increasing numbers of people in the emerging nations being able to afford industrialized energy and consumption patterns.

Together with significant changes in the energy supply structure such as higher shares of renewable energies, this all will lead to significant changes in the energy system.

As the provision of energy to modern societies needs heavy investment in long living infrastructures such as electricity grids, power plants, wind farms, coal mines, oil and gas fields, pipelines, and terminals, many stakeholders such as oil giants (Shell, BP), governments (US Department of Energy), international agencies (IEA,

IRENA), or NGOs (Greenpeace) as well as academic literature provide studies on the future of the energy system, often looking as far ahead as the middle of the century.

An increasing number of these energy scenario studies or energy outlooks has been concerned with analyzing and describing how future energy systems could be shaped in order to drastically reduce greenhouse gas emissions from the use of fossil fuels and sometimes also to achieve other sustainability targets.

Figure 5 condenses the results of a large number of such scenario studies for six major groups of GHG emitters: CO_2 emissions from transport, buildings, industry, electricity generation, as well as agriculture, forestry, and land use and all other GHG emissions from all sectors. Aggregated results are given for 2010 as a base year and the scenario years 2030, 2050, and 2100. The figure shows that the so-called baseline scenarios project strongly increasing emission growths for all segments apart from agriculture, forestry, and land use, where baselines expect slight decreases. The

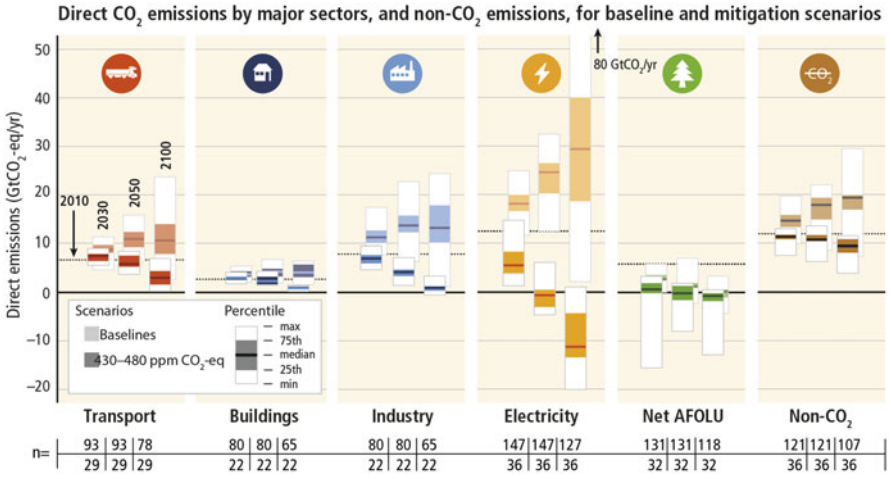


Fig. 5 Direct CO_2 emissions by major sectors, and non-CO_2 emissions, for baseline and mitigation scenarios. Carbon dioxide (CO_2) emissions by sector and total non-CO_2 greenhouse gases (Kyoto gases) across sectors in baseline (faded bars) and mitigation scenarios (solid color bars) that reach about 450 (430–480) ppm CO_2-eq concentrations in 2100 (likely to limit warming to 2 °C above preindustrial levels). Mitigation in the end-use sectors leads also to indirect emission reductions in the upstream energy supply sector. Direct emissions of the end-use sectors thus do not include the emission reduction potential at the supply side due to, for example, reduced electricity demand. The numbers at the bottom of the graphs refer to the number of scenarios included in the range (upper row, baseline scenarios; lower row, mitigation scenarios), which differs across sectors and time due to different sectoral resolution and time horizon of models. Emission ranges for mitigation scenarios include the full portfolio of mitigation options; many models cannot reach 450 ppm CO_2-eq concentration by 2100 in the absence of carbon dioxide capture and storage (CCS). Negative emissions in the electricity sector are due to the application of bioenergy with carbon dioxide capture and storage (BECCS). "Net" agriculture, forestry, and other land-use (AFOLU) emissions consider afforestation, reforestation, as well as deforestation activities. (Source: IPCC 2014b, Figure SPM.14 with kind permission of IPCC Secretariat, Web Information and Publications)

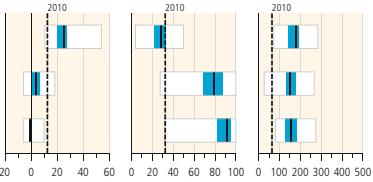
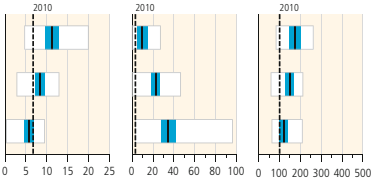
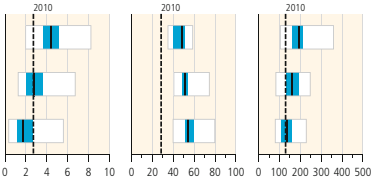
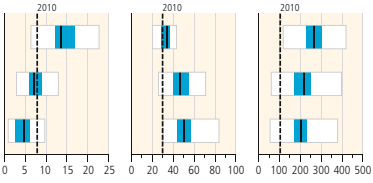
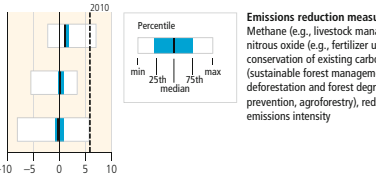
baseline scenarios are often called "business-as-usual" scenarios, although one might question if such developments would really occur as usual business until 2100, given the strong growth in GHG emissions and thus effects on climate change that these baselines imply. The collection of baselines by the IPCC shows an unmitigated growth of emissions until 2100 with a median increase of roughly two thirds between 2010 and 2100. Such growth in energy use and GHG emissions would lead to global mean temperature increases of around 4 °C or even more by the end of the century with significant related consequences for global ecosystems and human development. Countries all over the world have already committed themselves to reduce their GHG emissions significantly over the coming years (see section "Global Regulation of Greenhouse Gas Emissions") in order to move from baseline to emission trajectories in line with the global climate targets.

Figure 5 covers such climate mitigation scenarios, i.e., scenarios that achieve maximum global atmospheric concentrations of greenhouse gases between 430 and 480 parts per million (ppm). With such concentrations, most climate models expect global mean surface warming rates of 1.5–2 °C. The figure shows that the mitigation scenarios describe completely different trajectories than the baseline scenarios. Instead of strong growth trends in emissions, they display strongly declining emissions in all sectors with the deviations starting significantly before 2030 (or in other words, now). The difference is most pronounced for the currently largest emitter, the electricity sector. Instead of more than doubling between 2010 and 2100, emissions in the sector decline to zero by 2050 and are negative by 2100. But also in industry, transports, and buildings, emissions are reduced to levels close to zero in the median of all scenarios. Only emissions of non-CO_2 greenhouse gases remain quite stable, and these gases represent the largest emitter of GHGs already by 2030 and are responsible for the vast majority of emissions by 2100.

These differences in the emission segments mainly depend on the different technological options to reduce energy demand and GHG emissions. Table 2 gives an overview per emission sector for the year 2050. Deviating from Fig. 5, non-CO_2 GHGs are accounted for in the sector in which they occur (and not displayed as a separate segment). It can be seen that two factors are dominating GHG emissions: the final energy demand and the share of low carbon fuels in the sector. With declining energy demand and higher shares of low carbon fuels (or typically renewable energies), greenhouse gas emissions from the sectors can be reduced while they still provide (typically increasing) energy services to societies. The sectors, however, show significantly differing mitigation pathways. The final energy demand for electricity grows strongly in the baselines as well as the mitigation scenarios, but the share of low carbon fuels is up to almost 100% in the mitigation scenarios, while the baselines show even a small decline vs. 2010. These values reflect the characteristic of the electricity system as not being a final energy consumer. Instead, increasing amounts of low-carbon electricity will be used in transport, industry, and buildings providing these sectors indirectly with low carbon fuels. In transport and buildings, the mitigation scenarios see high increases in energy efficiency which compensate for a growth in energy service demand so that final

Table 2 Sectoral CO_2 emissions, related energy system changes, and examples for sectoral mitigation measures

Sectoral CO_2 emissions and related energy system changes

Sector	CO_2 emission ($GtCO_2$, 2050)	Low-carbon fuel share (%, 2050)	Final energy demand (EJ, 2050)
Energy supply[a]			
Transport			
Building			
Industry			
AFOLU			

Examples for sectoral mitigation measures

Key low-carbon energy options	Key energy saving options	Other options
Renewables (wind, solar bioenergy, geothermal, hydro, etc.), nuclear, CCS, BECCS, fossil fuel switching	Energy efficiency improvements of energy supply technologies, improved transmission and distribution, CHP and cogeneration	Fugitive CH_4 emissions control
Fuel switching to low-carbon fuels (e.g., hydrogen/electricity from low-carbon sources), biofuels	Efficiency improvements (engines, vehicle design, appliances, lighter materials), modal shift (e.g., from LDVs to public transport or from aviation to HDVs to rail), eco-driving, improved freight logistics, journey avoidance, higher occupancy rates	Transport (infrastructure) planning, urban planning
Building integrated RES, fuel switching to low-carbon fuels (e.g., electricity from low-carbon sources, biofuels)	Device efficiency (heating/cooling systems, water heating, cooking, lighting, appliances), systemic efficiency (integrated design, low/zero energy buildings, district heating/cooling, CHP, smart meters/grids), behavioural and lifestyle changes (e.g., appliance use, thermostat setting, dwelling size)	Urban planning, building lifetime, durability of building components and appliances, low energy/GHG intensive construction and materials
Process emissions reductions, use of waste and CCS in industry, fuel switching among fossil fuels and switch to low-carbon energy (e.g., electricity) or biomass	Energy efficiency and BAT (e.g., furnace/boilers, steam systems, electric motors and control systems, (waste) heat exchanges, recycling), reduction of demand for goods, more intensive use of goods (e.g., improve durability or car sharing)	HFC replacement and leak repair, material efficiency (e.g., process innovation, re-using old materials, product design, etc.)

Emissions reduction measures: Methane (e.g., livestock management), nitrous oxide (e.g., fertilizer use), conservation of existing carbon pools (sustainable forest management, reduced deforestation and forest degradation, fire prevention, agroforestry), reduction in emissions intensity

Sequestration options: Increasing existing carbon pools (e.g., afforestation, reforestation, integrated systems, carbon sequestration in soils)

Substitution options: Use of biological products instead of fossil/GHG intensive products (e.g., bioenergy, insulation products)

Demand-side measures: Reduction of loss and waste of food, changes in human diets, use of long-lived wood products

Source: Table 4.4 from IPCC, 2014: Topic 4: 4 Adaptation and Mitigation. In: Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland

energy use in these sectors remains roughly stable. In industry however, final energy demand grows only slightly slower in the mitigation scenarios as compared to the baseline scenarios. In a median, low-carbon energy shares achieve over 50% of

GHG emission reductions in buildings and industry and slightly less in transport. What it would take globally to achieve the mitigation scenarios will be described in more detail below.

What Would It Take to Stay Within the Limits of the Climate System?

As discussed in previously, the international community had debated an upper limit of 2 °C warming above preindustrial levels as being the maximum that diverse ecosystems and life-supporting functions of the planet could cope with and at which there would be a fair chance that most tipping points of the climate system would not be reached. Newer scientific results have shown, however, that to really avoid catastrophic changes, the threshold should rather be set at 1.5 °C. Politically, a number of small island states also pushed strongly to reduce the targeted upper limit of global warming, as it became evident that most of them would vanish by the end of the century due to sea level rise if temperatures increased by 2 °C. Therefore, the international community tasked the IPCC to develop a report that shows what would be needed to keep global temperature rise below 1.5 °C.

As the world has failed to reduce GHG emissions so far (Höhne et al. 2020), it is now very ambitious (Fig. 4 above), which makes it clear that global mean surface temperatures might reach this threshold in this decade already.

Figure 6 again shows a collection of mitigation scenarios from recent literature. The scenarios given are all compatible with the 1.5 °C target. The comparison of the scenarios shows the sooner and the faster emissions decline now, the easier the targets can be achieved in the long run. The low energy demand (LED) scenarios (SR15, p110) show an immediate and sharp decline of energy demand and emissions from 2020 on and achieve global zero CO_2 emissions by about 2050. As not all sectors can reduce that fast, some negative emissions (e.g., via bioenergy use with capturing and storing of the carbon) are assumed to compensate for the remaining GHG emissions.

The other scenario types are variations of this pattern but assume different energy system pathways. Emissions decline later or even still rise for a small number of years. In these scenarios, temperatures will "overshoot" the 1.5 °C threshold for some decades and decline later in the century, back to 1.5 °C. In addition, already from 2040 onward, very significant amounts of negative emissions would be necessary to compensate for the remaining emissions and much more for the emission overshoot accumulated between 2020 and 2050. It is, however, highly contested if negative emissions of such an immense volume would even be technologically feasible, let alone what their costs would be.

All scenarios show that in order to achieve the 1.5 °C target, the world needs to stop increasing emissions almost now and has to reduce them at very high and unprecedented annual rates for the coming decades. The reason for this is simple. As the amount of cumulative emissions that is compatible with the 1.5 °C limit, the "global climate budget," is limited, the mathematics are very clear: if emissions still rise instead of going down, this requires much faster action later. Governments around the globe were not able to stop emission growths over the last almost three

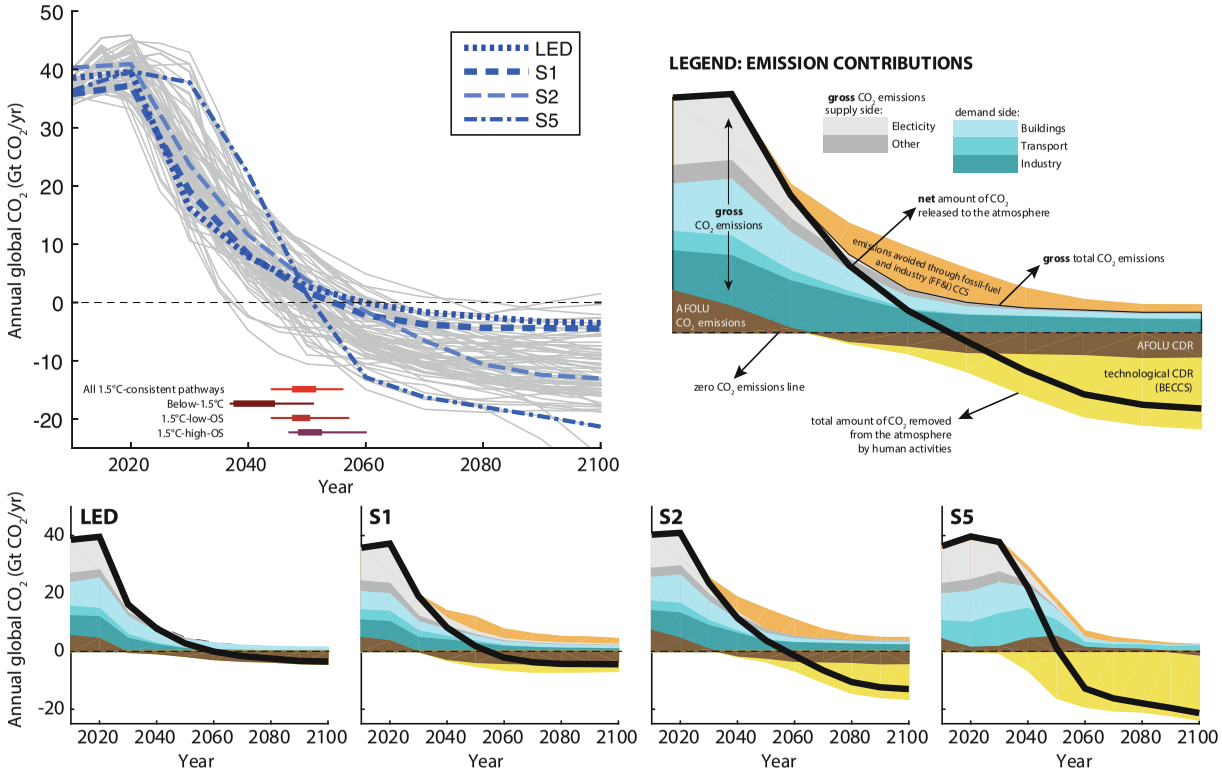


Fig. 6 Evolution and breakdown of global anthropogenic CO_2 emissions until 2100. The top-left panel shows global net CO_2 emissions in below 1.5 °C, 1.5 °C low overshoot (OS), and 1.5 °C high OS pathways, with four illustrative 1.5 °C-consistent pathway archetypes highlighted. Ranges at the bottom of the top-left panel show the 10th–90th percentile range (thin line) and interquartile range (thick line) of the time that global CO_2 emissions reach net zero per pathway

decades. Therefore, the available emission budget has already been almost fully used and every year with current or even higher emissions will consume a disproportionate share of the remaining budget. This means that reductions in the years to come have to be even more pronounced.

Figure 7 shows the result of these facts. Achieving the 1.5 °C target therefore means that the strategies depicted to reduce emissions have to start very early and have to be significantly accelerated as compared to the 2 °C scenarios. Figure 8 in the next subsection is even more telling as it shows the related global investments in the energy system.

The Economics of GHG Emissions and Their Mitigation

The climate mitigation scenarios discussed above assume significantly different investment trajectories as seen in the past or expected in a "business-as-usual" future. This is in itself a challenge, not only because of the volume of investments which is moderately above historic values, but mainly because investments in some sectors such as fossil generation see a massive decline, while investments in others such as renewable energies or energy efficiency need to be ramped up massively.

Figure 8 explores details on the investments necessary to convert energy systems around the globe. It compares historical with future (2016–2050) annual investments in different segments of the global energy system for different scenarios. These are baseline scenarios (see above), NDC scenarios which describe what would happen if governments around the globe implemented their emission reduction pledges under the Paris Agreement (see section "Regulation After 2020 Under the Paris Climate Agreement"), 2 °C scenarios, and 1.5 °C scenarios. The first result from the figure is that all the changes in the energy system indicated above would not result in significantly larger volumes of investment in the global energy system. Investment totals as modelled increase from 2300 billion US $ per year in the baselines, which is roughly comparable or up to 10% higher than historic investment volumes, to slightly above 3000 billion US $ per year in the 1.5 °C scenarios. This would be an increase in investment of less than a third vs. baselines, but given the highly uncertain ranges, it is also possible that the difference would be significantly smaller.

Next to moderately ramping up global investments to make the energy systems climate compatible, there are huge changes necessary regarding which investments are made. Investments into the so far largest segment of emissions, fossil fuel extraction and conversion, are cut by half in the 2 °C and by two thirds in the

Fig. 6 (continued) class and for all pathway classes combined. The top-right panel provides a schematic legend explaining all CO_2 emission contributions to global CO_2 emissions. The bottom row shows how various CO_2 contributions are deployed and used in the four illustrative pathway archetypes (LED, S1, S2, S5. Note that the S5 scenario reports the building and industry sector emissions jointly. Green-blue areas hence show emissions from the transport sector and the joint building and industry demand sector, respectively. (Source: Rogelj et al. 2018, Figure 2.5 with kind permission of IPCC Secretariat, Web Information and Publications)

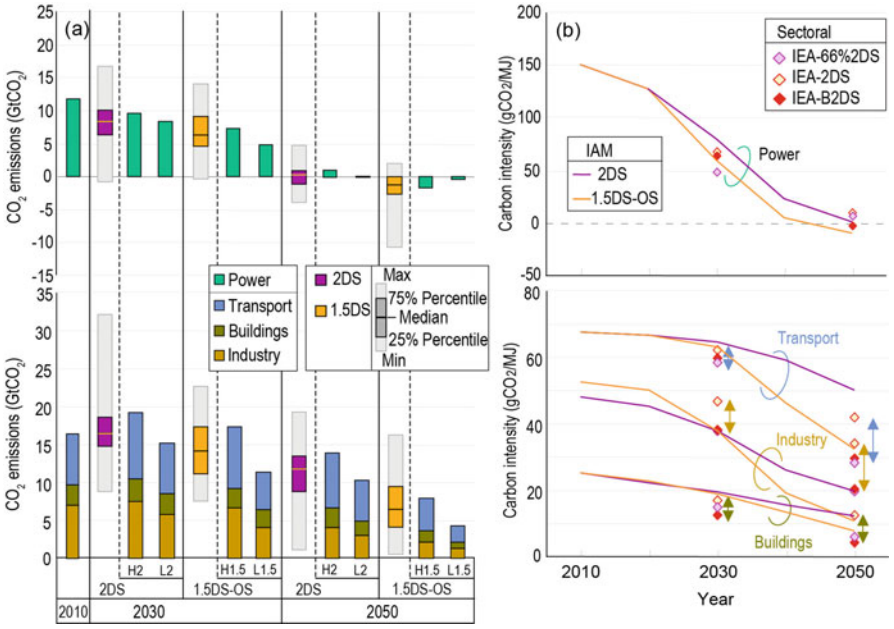


Fig. 7 Comparison of (**a**) direct CO_2 emissions and (**b**) carbon intensity of the power and energy end-use sectors (industry, buildings, and transport sectors) between Integrated Assessment Models (IAMs) and sectoral studies by IEA and IRENA. Diamond markers in panel (**b**) show data for IEA-ETP scenarios (2DS and B2DS) and IEA/IRENA scenario (66% 2DS). Note: for the data from IAM studies, there is rather large variation of projections for each indicator. (Source: Rogelj et al. 2018, Figure 2.20 with kind permission of IPCC Secretariat, Web Information and Publications)

1.5 °C scenarios. Investments in the fossil electricity generation without carbon capture and storage (CCS) will have to be almost completely stopped soon, even for the 2 °C scenarios. Instead investments in renewable energies, energy efficiency, and other low-carbon generation options have to be at least doubled between the baselines and the 1.5 °C scenarios, and investments into the electricity system also need to be significantly increased.

Having in mind that these changes need to occur almost immediately, the dimensions of the challenge become clearer. The massive decline in fossil investment creates of course massive challenges for regions and companies now living from fossil fuel extraction and conversion. These range from traditional coal regions in many countries all over the world, via the big oil and also gas-producing countries, whose economies often are dependent on the revenues of their fossil resources. Also international maritime trade with around 50% of the tonnage being fossil fuels, port, and logistic businesses as well as refineries are all affected. Builders and developers of fossil power plants will lose a large share of related businesses. On the other hand, ramping up global renewable energy, energy efficiency, and other low carbon as well

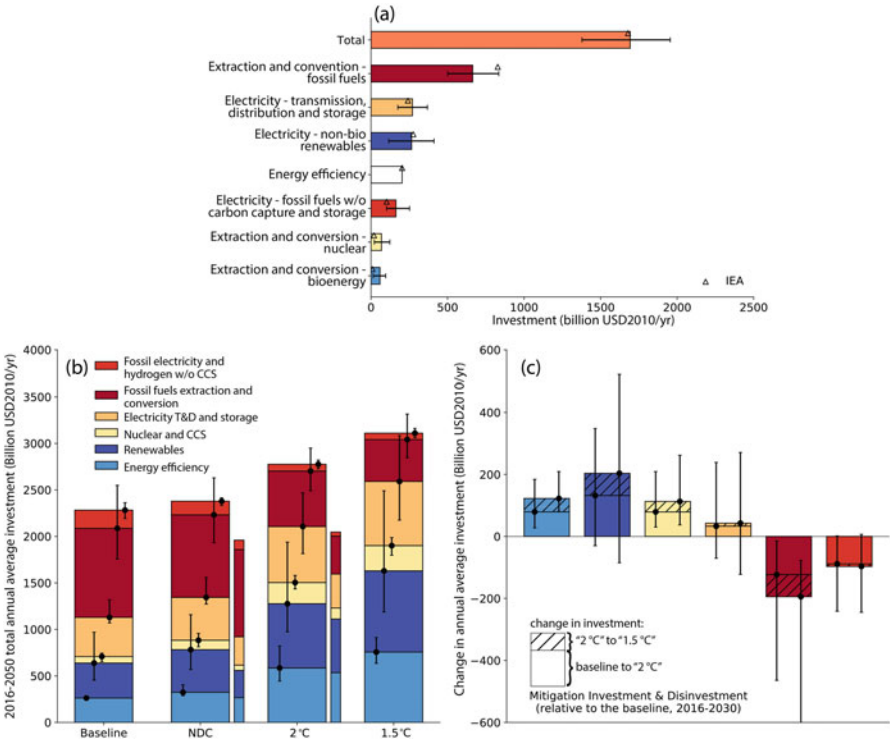


Fig. 8 Historical and projected global energy investments. (**a**) Historical investment estimates across six global models from (McCollum et al. 2018) (bars = model means, whiskers full model range) compared to historical estimates from the IEA (International Energy Agency (IEA) 2016) (triangles). (**b**) Average annual investments over the 2016–2050 period in the "baselines" (i.e., pathways without new climate policies beyond those in place today), scenarios which implement the NDCs ("NDC," including conditional NDCs), scenarios consistent with the lower 2 °C pathway class ('2 °C'), and scenarios in line with the 1.5 °C low overshoot pathway class ("1.5 °C"). Whiskers show the range of models; wide bars show the multimodel means; narrow bars represent analogous values from individual IEA scenarios (OECD/IEA and IRENA 2017). (**c**) Average annual mitigation investments and disinvestments for the 2016–2030 periods relative to the baseline. The solid bars show the values for "2 °C" pathways, while the hatched areas show the additional investments for the pathways labelled with "1.5 °C." Whiskers show the full range around the multimodel means. T&D stands for transmission and distribution, and CCS stands for carbon capture and storage. Global cumulative carbon dioxide emissions, from fossil fuels and industrial processes (FF&I) but excluding land use, over the 2016–2100 timeframe range from 880 to 1074 GtCO2 (multimodel mean: 952 GtCO2) in the "2 °C" pathway and from 206 to 525 GtCO2 (mean: 390 GtCO2) in the "1.5 °C" pathway. (Source: Rogelj et al. 2018, Figure 2.27 with kind permission of IPCC Secretariat, Web Information and Publications)

as electricity system investment also poses massive challenges to governments and investors. Current markets still fail largely in creating appropriate conditions to make these investments viable, which means that a large (but declining) share of the investments needed rely strongly on public support.

Particularly the loosing regions and sectors, who are typically well-established players for whom the fossil assets are an important part of their (economic) power, will most probably not easily buy in to such scenarios. The discussions around coal phase out in many countries provide telling examples but are only the tip of the iceberg if it comes to implementing 1.5 °C scenarios. On the other hand, it is extremely difficult to double economic activities in clean energy investment virtually overnight. New forms of political cooperation might be needed to achieve ramp-up rates and cost decreases strong enough to achieve the target.

The economic consequences of climate change, however, are much wider than the changes in energy system investments depicted above. Climate change will also cause significant economic damages due to losses in agricultural yields, higher costs in water supply, and damages due to climate-related weather events such as stronger storms, floods, etc. (see section "Climate Damages and Tipping Points: Why Is It Necessary to Limit Global Warming?"). While in the past there had been a discussion if warming-related benefits could compensate for these losses on a global level, nowadays the analyses seem to agree that climate damages will significantly outpace benefits, if these occur at all.

Global Regulation of Greenhouse Gas Emissions

Although the greenhouse effect and the role energy use plays were known since the end of the nineteenth century, it took until the 1990s to establish a political response globally. In 1992, the UN established the Framework Convention on Climate Change (UNFCCC) as the global level legal framework for regulating greenhouse gas emissions and the fight against climate change. Under this framework, an intensive global climate diplomacy has emerged since then. Since 1990, climate policy has seen three phases of regulation of greenhouse gas emissions as well as climate mitigation and adaptation. Each of these phases is described in one of the following subsections. Finally, the section is concluded by a subsection on the Sustainable Development Goals which complement the climate regulation with regard to other fields of global sustainability.

The UN Framework Convention on Climate Change (UNFCCC)

In 1990, the UN General Assembly decided to start negotiations on an international treaty on climate change. After 2 years of negotiations, the result – the United Nations Framework Convention on Climate Change (UNFCCC) – was adopted in the framework of the United Nations Conference on Environment and Development (UNCED), the so-called Earth Summit, in Rio de Janeiro in 1992. The UNFCCC entered into force on 21 March 1994, 90 days after it had been ratified by 50 states. To date, 197 states – nearly all countries in the world – are Parties to the UNFCCC (UNFCCC Website 2020b).

The UNFCCC is the constitution of international climate policy, establishing its main goals, principles, and institutions. Article 2 establishes its central objective: to prevent dangerous anthropogenic interference with the climate system "within a timeframe that is sufficient to allow ecosystems to adapt naturally to climate change, to ensure that food production is not threatened and to enable economic development to proceed in a sustainable manner." However, the Convention did not define which level of climate change was considered to be "dangerous." This question was resolved only nearly 20 years later with the adoption of the 2 °C limit (see subsequent sections).

One of the key principles of the Convention is that industrialized and developing countries have common but differentiated responsibilities and that industrialized countries are supposed to take the lead in combating climate change (Article 3.1). This principle is intended to take into account the fact that industrialized countries have already emitted high amounts of GHGs since the beginning of industrialization and are still responsible for high per capita emissions and high absolute emissions today. The Convention therefore divides the countries of the world into Annex I Parties on the one hand and non-Annex I Parties on the other. Annex I of the Convention lists the traditional industrialized countries and the former Eastern bloc countries with economies in transition. Annex I Parties are required to implement policies and measures on the mitigation of climate change by limiting GHG emissions and protecting and enhancing GHG sinks and reservoirs, with the aim to return their CO_2 emissions to 1990 levels (Article 4.2). In addition, they committed to supporting developing countries financially, technologically, and with capacity building (Article 4.3). On the other side, the Convention explicitly conditions the extent to which developing countries implement their commitments on the extent to which developed countries provide financial and technological support (Article 4.7).

Furthermore, to provide transparency on implementation and progress made, all Parties committed to regularly providing national inventories of GHG emissions and removals by sinks as well as a description of steps taken to implement the Convention (Article 12). Content requirements and timetables for these "national communications" were differentiated for Annex I and non-Annex I Parties, developed out in detail in subsequent years, and repeatedly tightened. The progressive tightening of the transparency requirements culminated in the "Enhanced Transparency Framework" established by the Paris Agreement in 2015 (see below).

Article 7 of the UNFCCC establishes annual meetings. The task of the Conference of the Parties to the Convention (COP) is to review the implementation of the Convention and related legal instruments and to take decisions necessary to ensure the implementation of the Convention. Articles 9 and 10 establish two subsidiary bodies to the COP: the Subsidiary Body for Scientific and Technological Advice and the Subsidiary Body for Implementation. While the COP usually meets annually, the subsidiary bodies usually meet two times per year. Furthermore, Article 8 of the Convention establishes a permanent Secretariat to support its implementation.

Regulation from 1995 to 2012 Under the Kyoto Protocol to the UNFCCC

While the UNFCCC established the framework for international climate policy, it does not contain any specific commitments to act against climate change. The intention was that more specific commitments should be established by subsequent legal instruments. Accordingly, the first COP (COP1), which met in Berlin in 1995, 1 year after the Convention had entered into force, adopted the so-called Berlin Mandate to negotiate a protocol to the Convention (Oberthür and Ott 1999).

The negotiations initiated in Berlin were concluded with the adoption of the Kyoto Protocol at COP3 in Kyoto, Japan, in 1997. The negotiations had been hampered by a fundamental North-South conflict. The main controversy had been around countries' respective responsibilities for climate change and which commitments they should undertake correspondingly. While the developing countries wanted to discuss binding commitments only for the industrialized countries in accordance with the Berlin Mandate, the USA in particular emphasized that the new treaty could and should also address binding emission targets for developing countries (Oberthür and Ott 1999).

In the final outcome, only the Parties listed in Annex I to the Convention undertake to reduce their collective GHG emissions by at least 5 percent compared to 1990 levels between 2008 and 2012 (first commitment period of the KP) (Article 3.1). This commitment refers to a "basket" of six GHGs and groups of GHGs listed in Annex A of the Protocol:

- Carbon dioxide (CO_2).
- Methane (CH_4).
- Nitrous oxide (N_20).
- Hydrogenated fluorocarbons (HFCs).
- Perfluorinated fluorocarbons (PFC).
- Sulfur hexafluoride (SF_6).

The aggregate reduction commitment of 5% refers to the GWP of these gases over a 100-year period (see above).

Each Annex B Party has its own emission target, called quantified emission limitation and reduction commitment listed in Annex B to the KP. Annex B comprises all industrialized countries listed in Annex I to the Convention, minus Belorussia and Turkey. The European Union (at that time still the European Community) has a special role; it acts in concert and has a collective target listed in Annex B.

In addition to the commitment to emission targets, the other main feature of the KP is the introduction of emission trading mechanisms to help Parties to meet their commitments, the so-called flexible mechanisms. These include:

- Emission trading among Parties, i.e., the purchase and sale of emissions units, which is regulated in Article 17 of the Protocol.

- "Joint Implementation (JI)," the implementation of climate protection projects among Annex B Parties, which is regulated in Article 6
- The "Clean Development Mechanism (CDM)," implementation of climate protection projects in developing countries, which is regulated in Article 12.

Four more years of negotiations were required to flesh out the implementation details for the KP. These negotiations were finally concluded at COP7 in Marrakech in 2001 with the adoption of the "Marrakech Accords." However, in the meantime, the USA had formally withdrawn from the Protocol. This posed a problem for the entry into force of the KP, which required ratification by at least 55 countries that together accounted for at least 55 percent of Annex I CO_2 emissions in the base year (1990). The USA alone accounted for more than one third of this value, in their absence the entry into force required ratification by most other Annex B countries, in particular Russia (Walsh 2004). Nonetheless, the threshold was crossed on 4 November 2004, and the Protocol entered into force on 16 February 2005, 90 days later (UNFCCC Website 2020a).

In 2012, the KP lost another Annex B Party. Instead of reducing its emissions, Canada's emissions had increased substantially. In order to avoid the penalties envisaged in the KP, Canada therefore withdrew its participation (The Guardian & Agencies 2011).

The remaining Annex B Parties complied with their emission targets. However, it has to be noted that compliance was greatly helped by (a) the severe economic contraction in the former Eastern bloc countries in the 1990s and (b) the financial crisis that started in 2008 and severely reduced economic output around the world (Shishlov et al. 2016).

Regulation Till 2020 Under the Cancún Agreements

Following the entry into force of the Protocol, the annual climate conferences were no longer only the Conference of the Parties to the Framework Convention on Climate Change (COP), but also the "Conference of the Parties serving as Meeting of the Parties to the Kyoto Protocol" (CMP). The first COP/CMP met in Montreal in 2005. In addition to taking further decisions on the implementation of the UNFCCC and KP, the conference also started new rounds of talks on binding commitments for "post-2012," i.e., the period after the first commitment period of the Kyoto Protocol (Wittneben et al. 2006).

However, industrialized countries were not willing to envisage a second Kyoto period without enhanced action from developing countries. In addition to the Kyoto negotiations, the Montreal conference therefore also started a second negotiating stream to enhance action under the Convention. While developing countries were at first opposed to engaging more strongly in GHG reductions, COP13 in Bali in December 2013 marked a breakthrough. In the "Bali Action Plan (BAP)" adopted at COP13, developing countries agreed to undertake "nationally appropriate mitigation actions (NAMAs)" in the context of

sustainable development, contingent on enhanced financial, technological, and capacity building support from industrialized countries. Another section of the BAP covered adoption of emission targets for developed countries, with the intention to include the USA in the new arrangements, which continued to abstain from the Kyoto Protocol (Watanabe et al. 2008).

The new arrangements were supposed to be adopted at COP15 in Copenhagen in 2009. However, COP15 failed to deliver on the expectations. Marked by logistical failures and political confusion in the final days of the conference, negotiations among a limited number of Parties behind closed doors produced a "Copenhagen Accord." In the Accord, countries for the first time agreed on the long-term target to limit global temperature increase to at most 2 °C above preindustrial levels. The Accord also contained provisions for the registration of emission targets of developed countries, NAMAs of developing countries, and enhanced support from developed to developing countries. In particular, industrialized countries pledged to increase the provision of climate finance to USD 100 billion annually by 2020 (UNFCCC 2010).

However, the Accord was not accepted by the plenary of the COP as many countries complained about its lack of ambition and the closed process which had produced the Accord (Sterk et al. 2010).

Subsequent negotiations were able to pick up the pieces. COP16 in Cancún, Mexico, yielded the "Cancún Agreements (CA)," a lengthy COP decision that set a new framework for enhanced climate action until 2020. It confirmed and further fleshed out the elements that had been contained in the Copenhagen Accord, in particular the 2 °C limit, provisions for emission targets of developed countries, and NAMAs of developing countries. In addition, the CA provided for the establishment of a Green Climate Fund (GCF) and of a Technology Mechanism to enhance support for developing countries. The CA also strengthened the requirements for how often and in how much detail Parties need to report on their GHG emissions and climate actions they are taking (UNFCCC 2011).

However, the CA are only a COP decision, not a treaty, and hence have relatively limited legal standing. In addition, the future of the Kyoto Protocol was still open. In the course of the negotiations up to Cancún, most industrialized countries had adopted a position of wanting a new comprehensive agreement covering all countries, instead of continuing on two tracks, with the KP covering industrialized countries and another agreement covering the rest of the world. COP17 in Durban, South Africa, in 2017 therefore started another round of negotiations. The EU agreed to go ahead with a second commitment period under the KP in order to satisfy the demands of developing countries. In return, all countries agreed to start negotiations on a new comprehensive agreement covering all countries from 2020 onward (Sterk et al. 2011).

COP18 in Doha, Qatar, adopted the "Doha Amendment" which contains the details for the second Kyoto Period. However, several of the countries that had undertaken commitments in the first commitment period did not offer new commitments for the second one, in particular Japan and Russia (Sterk et al. 2012).

Regulation After 2020 Under the Paris Climate Agreement

After 25 years of international climate negotiations, in 2015, COP21 in Paris finally adopted a universal agreement requiring climate action from all countries. The necessary ratifications came together at nearly unprecedented speed; the Agreement entered into force already on 4 November 2016 (UNFCCC Website 2020b).

The Agreement has three long-term objectives. First, the Agreement strengthens the long-term temperature limit. Whereas the Cancún Agreements had set down the objective to stabilize temperature increase below 2 °C, the Paris Agreement aims at "well below" 2 °C and to make "best efforts" to stay below 1.5 °C (Article 2.1(a)). This strengthening of the temperature limit was due to the insistence of small island states and least developed countries, which are particularly vulnerable to the impacts of climate change.

The Paris Agreement also stipulates what achieving the temperature limit will need to mean in terms of emission pathways: according to Article 4.1, global emissions are to peak as soon as possible; and a balance between emissions of GHGs from sources and GHG removal by sinks is to be achieved in the second half of the century.

The second long-term objective of the Agreement is to increase the ability to adapt to the adverse impacts of climate change and foster climate resilience and low greenhouse gas emissions development (Article 2.1(b)). The third objective is to make all financial flows consistent with low greenhouse gas emissions and climate-resilient development (Article 2.1(c)).

However, the adoption of this universal agreement came at a price: the Paris Agreement does not contain legally binding commitments for the individual countries, as is the case in the Kyoto Protocol. Instead, there is only a procedural obligation for countries to submit and maintain a "nationally determined contribution (NDC)" (see also section "What Would It Take to Stay Within the Limits of the Climate System?"). There are no international requirements on the format and content of the NDCs. The agreement only suggests that developed countries should undertake to define economy-wide absolute emission reduction targets as in the Kyoto Protocol, whereas developing countries "are encouraged to move over time towards economy-wide emission reduction or limitation targets" (Article 4.3). NDCs are supposed to reflect Parties "highest possible ambition," but there is no guidance as to what this is supposed to mean. There also is no requirement for Parties to actually achieve their NDCs, only a requirement to pursue domestic measures toward this end (Article 4.2).

As result of this lack of formal requirements, the traditional industrialized countries have all submitted Kyoto-style absolute emission targets, whereas developing countries feature a variety of relative emission targets indexed to GDP or framed in a comparison to a business as usual scenario. Many countries with low capacity have notified no emission targets whatsoever. Instead, their NDCs contain lists of specific policies and measures they intend to undertake. Furthermore, the target dates of the NDCs vary. Some have 2025 as target date; the majority has 2030; some have even longer timeframes (WRI 2020).

Furthermore, the ambition of nearly all NDCs is much weaker than what would be necessary to achieve the temperature limits of the Paris Agreement. Instead of limiting temperature increase well below 2 °C or even 1.5 °C, the current NDCs would lead to warming of between 3 °C and 4 °C if not strengthened further. Moreover, in many countries, the policies implemented so far will not even deliver the reductions pledged in the NDCs (Roelfsema et al. 2020; UNEP 2019, p. 2019).

To counteract these weaknesses, the Paris Agreement contains a so-called ambition cycle. Parties are required to revise their NDCs or submit new NDCs every 5 years, for the first time in 2020, then 2025, etc. Furthermore, Parties will collectively evaluate their progress toward the objectives of the Paris Agreement, also every 5 years (Article 14). The results of these "Global Stocktakes (GST)" are to inform the revision of existing and elaboration of new NDCs. The first GST will take place in 2022/2023. The Paris Agreement thereby establishes a continuous series of political reflection and subsequent NDC revision. This mechanism is supposed to contribute to a continuous "ratcheting up" of ambition, to ultimately bring the NDCs in line with the achievement of the long-term objectives.

Another element that is supposed to compensate for the lack of legally binding commitments is the "enhanced transparency framework for action and support" under Article 13. The aim of the framework for *transparency of action* is "to provide a clear understanding" of climate action and to track progress, while the aim of the framework for *transparency of support* is "to provide clarity on support provided and received" and to provide an overview of aggregate financial support. In addition, the transparency framework is supposed to inform the Global Stocktake.

The PA removes the binary distinction between developed and developing countries that was established by the Convention and provides that all Parties shall account for their contributions (Article 4.13). To nonetheless account for the different circumstances of Parties, the transparency framework "shall provide flexibility (. . .) to those developing country Parties that need it in the light of their capacities" (Article 13.2).

In detail, Article 13 requires Parties to regularly provide national greenhouse gas inventories that need to be prepared using IPCC good practice guidance, as well as information necessary to track progress made in implementing and achieving NDCs. All Parties are required to submit this information at least every 2 years, with the exception of small island developing states and least developed countries (UNFCCC 2016, p. 90). Furthermore, Article 13 requires developed country Parties to report on financial, technology, and capacity building support provided by them to developing countries. All this information reported by Parties will undergo a technical expert review. The review is supposed to identify areas of improvement and to review the consistency of the information provided with the reporting requirements. Moreover, Parties will discuss each other's performance as part of a "facilitative, multilateral consideration of progress."

On climate finance, the COP decision adopting the PA reconfirms developed countries' commitment of mobilizing USD 100 billion in 2020 and beyond. In addition, Parties agreed that a new collective financing target will be adopted by 2025 (UNFCCC 2016, p. 53).

The 2018 climate conference in Katowice adopted detailed implementation regulations for most aspects of the PA. The Katowice conference also conducted a precursor to the Global Stocktake, named "Talanoa Dialogue." The outcome of the Talanoa Dialogue confirmed that the level of ambition of currently submitted NDCs is insufficient, but countries were not able to agree on a strong call to action (Obergassel et al. 2019).

As of the time of writing this text in the spring of 2020, it remains to be seen whether the PA's "ratchet" mechanism will work as intended. Parties are supposed to submit new or updated NDCs by 2020. The strengthening of NDCs is becoming ever more urgent. Climate change is caused by the accumulation of GHGs in the atmosphere over time. Despite 25 years of climate diplomacy, global GHG emissions are still growing. The longer it takes to halt and reverse this trend, the steeper the subsequent reduction will need to be. According to the IPCC special report on the 1.5 °C limit (see section "What Would It Take to Stay Within the Limits of the Climate System?"), balancing emissions from sources and removals by sinks in the second half of the century, as aimed for in the Paris Agreement, is actually no longer sufficient to keep the 1.5 °C limit within reach. Maintaining a good chance of achieving the 1.5 °C limit would require to essentially halve global CO_2 emissions by 2030 and reduce them to net zero by 2050 (IPCC 2018c).

Unfortunately, the USA repeated the performance they had given in relation to the Kyoto Protocol – walking away from a treaty the content of which they had determined to a large extent. In November 2016, about a year after the adoption of the Agreement in Paris, Donald Trump was elected as new US president. He had campaigned on ending national climate policy and withdrawing from the Paris Agreement and publicly announced the US withdrawal on 1 June 2017 (The White House 2017). Under President Obama, the USA had been a key driver of the adoption of the PA. It remains to be seen what progress the rest of the world will be able to make without the USA.

The Link to the UN Sustainable Development Goals (SDG)

2015 was a watershed for international sustainability governance. The international community adopted not only the Paris Agreement but also Agenda 2030 (United Nations 2015). The Agenda was developed in a participatory process involving governments, civil society, business, academia, the UN, and also individuals and was adopted by the UN General Assembly on 25 September 2015. It builds on the outcomes of the UN Conference on Sustainable Development (Rio + 20) in 2012 and the Millennium Development Goals (MDGs) and their targets, which refer to the year 2015. The Sustainable Development Goals (SDGs) reflect the global challenges to sustainable development and define goals to be achieved by 2030 (Fig. 9). In contrast to their predecessors, the Millennium Development Goals (MDGs), the SDGs refer not only to developing countries but to all countries of the world. They are to be understood as an overall system of global development goals. The SDGs are interdependent and inseparable.

Fig. 9 The UN Sustainable Development Goals, source and Copyright © United Nations, adapted by Wuppertal Institut, "The content of this publication has not been approved by the United Nations and does not reflect the views of the United Nations or its officials or Member States." https://www.un.org/sustainabledevelopment/sustainable-development-goals/

Nevertheless, they can be logically grouped into four categories. The SDGs are concretized by 169 sub-objectives and indicators, which enable an assessment of the achievement of objectives.

Both, the Agenda 2030 and the Paris Agreement stress the need for integration. In its preamble, the PA emphasizes "the intrinsic relationship that climate change actions, responses and impacts have with equitable access to sustainable development and eradication of poverty." Also, under the Agreement's Article 2, its objectives are to be achieved "in the context of sustainable development and efforts to eradicate poverty." The Agenda 2030, for its part, includes climate action as one of the Sustainable Development Goals (SDGs) to be achieved by 2030 and stresses the interlinkages between and the integrated nature of all SDGs.

Actions to fulfill one SDG may impact others in some way – often positively but also negatively. For example, transport relates to no less than 12 targets of 9 SDGs directly or indirectly (SLoCaT 2015). The largest share of transport emissions comes from motorized road transport. Minimizing road use, e.g., through optimized infrastructure and planning processes, shifting toward less-polluting travel modes (e.g., rail freight, public transport, bicycles, or walking) as well as improving fuel and vehicle efficiency yields many benefits that go beyond mere GHG abatement. For example, less vehicle use means less local air pollution and thus better public health. Active forms of transport such as walking and cycling have positive health effects as well.

The IPCC in its special report on the implementation of the 1.5 °C target comprehensively identifies potential synergies and trade-offs between climate protection measures and the SDGs in relation to sectoral portfolios of measures on energy demand, energy supply, and land use and their probability. Figure 10 shows that although there are risks of conflicting objectives, the potential for synergies is much greater. Both the

Indicative linkages between mitigation options and sustainable development using SDGs (The linkages do not show costs and benefits)

Mitigation options deployed in each sector can be associated with potential positive effects (synergies) or negative effects (trade-offs) with the Sustainable Development Goals (SDGs). The degree to which this potential is realized will depend on the selected portfolio of mitigation options, mitigation policy design, and local circumstances and context. Particularly in the energy-demand sector, the potential for synergies is larger than for trade-offs. The bars group individually assessed options by level of confidence and take into account the relative strength of the assessed mitigation-SDG connections.

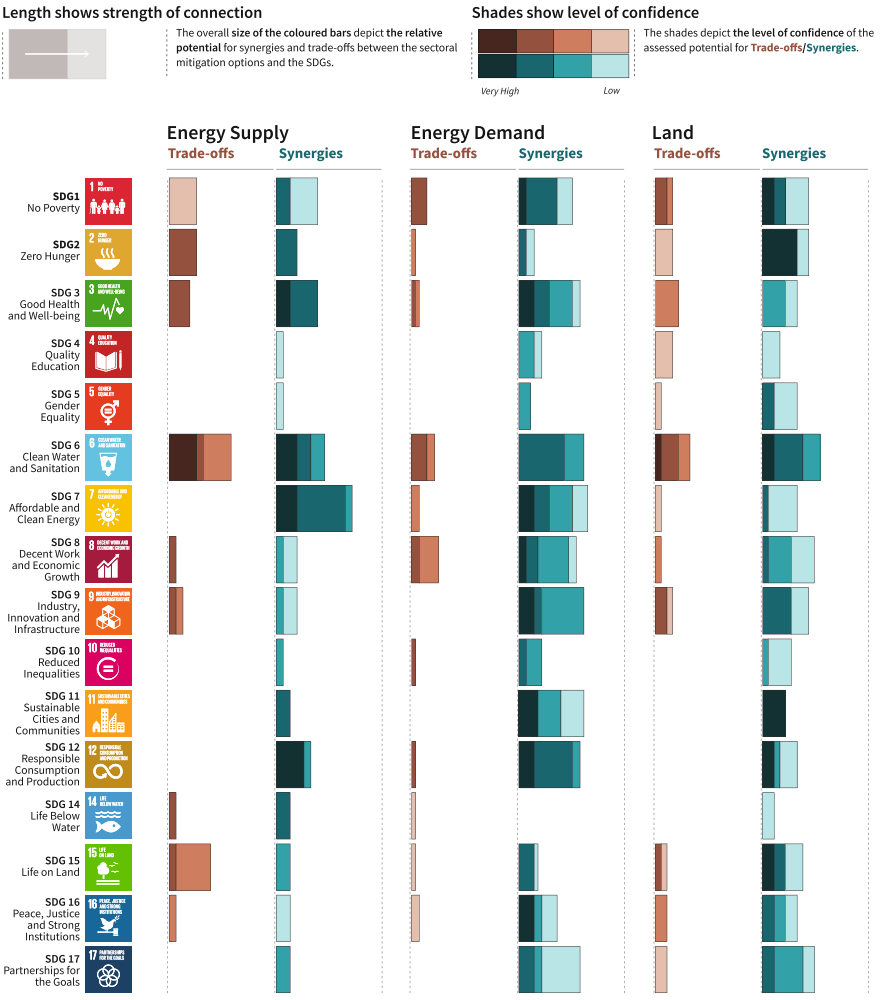


Fig. 10 Potential synergies and trade-offs between the sectoral portfolio of climate change mitigation options and the Sustainable Development Goals (SDGs). The SDGs serve as an analytical framework for the assessment of the different sustainable development dimensions, which extend beyond the time frame of the 2030 SDG targets. The assessment is based on literature on mitigation options that are considered relevant for 1.5 °C. The assessed strength of the SDG

mix of policy instruments used and the concrete design of the instruments and the strategy behind them as well as local circumstances determine the extent to which synergies can be realized and conflicts of objectives reduced and avoided.

The WHO Special Report "Climate Change and Health" emphasizes the links between health and climate change, pointing out that climate change and health risks often have the same causes, in particular air pollution resulting from the use of fossil fuels in the transport and energy sectors. If it is not possible to limit climate change, a deterioration of the health level in all regions of the world is to be expected, especially in urban regions. As a result of consistent climate protection measures to meet the goals of the Paris Agreement by 2050, more than one million lives could be saved annually by reducing air pollution alone. The same analysis shows that the value of health gains alone would be about twice as high as the costs of climate protection policies (WHO 2018).

The Great Transition to a Climate-Neutral Global Society

Sections "What Would It Take to Stay Within the Limits of the Climate System?" and "The Economics of GHG Emissions and Their Mitigation" show what a significant challenge achieving the target of a maximum global warming of 1.5 °C above preindustrial levels will be for all states and all parts of society globally. This target would mean that the world becomes almost completely climate-neutral as early as the middle of the century. Such a change would be as significant as the Industrial Revolution of the nineteenth century but much faster and with a clear directionality needed. How such significant changes may be perceived is discussed in the next section, followed by a discussion of the importance of the current situation with the Coronavirus crisis creating one of the largest economic downturns witnessed in the last centuries. We end with a brief description of the "European Green Deal," one of or even the first ambitious policy program that aims at really implementing the great transition toward climate neutrality, and by this it could – and should – be an example for many nations around the globe to take ambitious and integrated measures to align their economic and social developments with the needs of mitigating the global climate crisis.

The Concept of a Great Transition

The previous sections showed that the regulation and mitigation of greenhouse gases is a relatively young field of policy which has emerged only since 1990. The past

Fig. 10 (continued) interactions is based on the qualitative and quantitative assessment of individual mitigation options. (Source: IPCC 2018, Figure SPM.4 with kind permission of IPCC Secretariat, Web Information and Publications)

30 years, however, saw the field rapidly emerging on all political levels, and now it is one of if not the primary field of cross-cutting policy globally. Yet the challenges lying ahead if the global community wants to avoid catastrophic climate change are even bigger and would need unprecedented efforts globally and on all political, economic, and societal levels (IPCC 2018b). The huge changes necessary which affect basically all fields of economy and society have been compared in size, consequence, and radicality to be equivalent to the Neolithic Revolution, i.e., the emergence of agricultural society, and the Industrial Revolution, i.e., the conversion to an industrial society. The challenge to achieve a climate neutral society has been therefore called a "Great Transformation" (Hamann et al. 2014, p. 1 ff.). What is unique compared to previous transformations is that the Great Transformation has to be organized in order to be fast enough to cope with the challenges the climate system imposes on human society. Historical transitions in contrast emerged as gradual and evolutionary changes.

Nevertheless, the German Advisory Council on Global Change WBGU refers to the work by Karl Polanyi who showed that the uncontrolled market forces and innovation processes of the industrial revolution were only then accepted and controlled when they were embedded in new social and political norms such as democracy and the welfare state. The learning is that the Great Transition toward a climate-neutral and sustainable society also needs a new social contract. The basic feature of such a "new global social contract for a low-carbon and sustainable global economic system" is the joint responsibility of individuals, civil societies, states, the global community, economy, and science.

According to the WBGU, this new social contract is needed to create three types of cultures which are needed to cope with the three central challenges of the future:

- Ecological responsibility: Culture of attentiveness
- Democratic responsibility: Culture of participation
- Future responsibility: Culture of obligation toward future generations

The Coronavirus Crisis Recovery as Critical Also for Handling the Climate Crisis

Parallel to writing this chapter about the Coronavirus, the world has seen the emergence of a global pandemic not witnessed in equal size for over a century. Both the potential death toll of the pandemic, particularly in the poorer parts of the world, and the already unfolding consequences of the complete stop of most of the social life and a huge share of all economic activities around the globe will have significant consequences on the road mankind will take in the coming years.

On short notice, the lockdown of most activities is significantly reducing greenhouse gas emissions and pollution all around the globe and has lead to the first absolute drop in global GHG emissions for several decades. Given a longer-term perspective, currently many are seeing the states as well as the global

community at a clear crossroads. Preserving the economy during the lockdown and restarting it afterward will need immense government support, and governments around the globe, or at least in the more affluent parts of it, have announced expenditures of dimensions never seen before. The crisis recovery measures now might be the main factor deciding which direction the global economy will take over the coming years and possibly decades. Either policy-makers will be willing and able to use these immense public expenditures to invest into climate neutral and sustainable energy and production systems and societies, or these funds will largely be used to simply restore or reset economy and society to the status quo ante the crisis.

The first option is highly challenging as all decisions and investment are being made under high time pressure. It is necessary that governments and market actors find ways to invest into "future" proof technologies and systems for recovery. This means that globally quick investments of massive scale need to be directed toward energy efficiency, climate-neutral industrial processes, circular economy, renewable energy generation, clean energy infrastructures, public transport, and more resilient and climate friendly cities (see section "The Economics of GHG Emissions and Their Mitigation"). An investment boom of such unprecedented size could be able to create a strong directionality in the further development of societies and, by this, speed up many processes and lock in societal and economic trajectories into a direction toward climate neutrality. While many of these investments could be ramped up quickly, lacking institutional capacities, old cultures, and paradigms, lack of technology readiness or resistance against new infrastructures might impose significant barriers and slow down such investment into sustainable and climate neutral societies.

Particularly the perceived urgency to act fast to limit the economic effects of the crisis, together with the power of incumbent interests, may be a strong driver to merely invest along the lines of existing investment patterns, thereby restoring and conserving old fossil-based structures in the economy and society. In this case, the future trajectory would be locked into a "baseline" route for quite a number of years and possibly decades to come, as a business-as-usual recovery would result in a large stock of new high-emission technology and infrastructure assets. Moreover, as the massive recovery expenditures will sharply increase public debt, societies would lack the funds to quickly replace these new high-emission assets with low-emission ones.

The European Green Deal as an Integrated Regulatory Approach

In fall 2019, the new European Commission started its term with the grand plan of a "European Green Deal" and the aim to make Europe the first climate-neutral continent. This Green Deal – clearly inspired by Roosevelt's New Deal that had helped the USA to recover from the Great Depression of the 1930s – has the ambition to be a comprehensive program for Europe's future. By directly linking policies for economic development and innovation to policies for a sustainable and

climate neutral economy as well as to policies for a fair and just transition, it clearly combines the three dimensions of sustainable development, i.e., ecology, economy, and social sustainability. What is new about this grand plan is that it aims at really integrating those targets. This follows the learning, driven by climate change and other environment-related crises, that economic and social development can no longer be seen as antipodes to ecologic sustainability but all three have to be achieved jointly and that also the economic future of Europe depends to large extent on its success to become frontrunner of a climate neutral and circular economy. Climate change together with digitalization is perceived as the major global mega-trend and thus as the core driver for innovation.

To implement the European Green Deal, the European Commission has defined a set of policy areas that together create a comprehensive and integrated package of the following eight policy areas to be tackled in a coordinated manner (https://ec.europa.eu/info/strategy/priorities-2019-2024/european-green-deal_en):

- Clean energy/opportunities for alternative, cleaner sources of energy
- Sustainable industry/ways to ensure more sustainable, more environmentally respectful production cycles
- Building and renovating/the need for a cleaner construction sector
- Sustainable mobility/promoting more sustainable means of transport
- Biodiversity/measures to protect our fragile ecosystem
- From farm to fork/ways to ensure more sustainable food systems
- Eliminating pollution/measures to cut pollution rapidly and efficiently
- Climate action/making the EU climate neutral by 2050

Given the immense and urgent challenges to avoid catastrophic climate change and to make societies more sustainable as depicted in the previous chapters, such integrated and ambitious approaches are urgently needed in order to underpin ambitious climate targets as aimed at in the global process of raising the ambition. Therefore, the European Green Deal can be seen as a model also for other states to organize their policies toward ambitious climate goals. If climate mitigation is increasingly seen by the global community as a core source for innovation and economic well-being, there will be chances that states, societies, and companies will be able to deliver on the needed strategies to keep global temperature change below the critical levels of 1.5 °C.

Cross-References

► Green and Sustainable Chemistry as Regulatory Levers
► Medical Aspects of Traffic Exhaust Regulations
► Regulation of Agrochemicals
► Risk Communication: Challenges for Toxicologists and Other Risk Experts

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Regulation of Agrochemicals

87

Volker Mostert

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Abstract

Plant protection products (PPPs) have to be authorized prior to their placing on the market. PPPs may only contain active substances (or safeners and synergists) that have been previously approved. The approval process for active substances involves the evaluation of an extensive toxicological data set and results in the setting of reference doses and hazard-based classification for human health effects. Exclusion criteria preclude the approval of active substances that are carcinogenic, mutagenic, and toxic to reproduction, or that have endocrine-disrupting properties.

PPPs can be only authorized if they are efficacious and safe for human health and the environment. The human health risk assessment for PPPs is based on reference doses that represent safe exposure levels for active substances for operators and consumers. The reference doses are derived from no-effect levels observed in a battery of toxicological tests ranging from acute to chronic studies.

V. Mostert (✉)
extera, Langenfeld (Rhld.), Germany
e-mail: vmostert@extera-vm.com

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Active substance · Plant-protection product · Data requirements · Hazard assessment · Exposure assessment

Introduction

Agrochemicals are strictly regulated with regard to their potential human health effects. This is necessary because human exposure potentially occurs on many levels. Applicators may be exposed during use of plant protection products (PPPs), but especially the potential exposure of the public at large to residues of agrochemicals and their transformation products requires regulatory attention. Such residues may be present in food, air, or drinking water. For this reason, PPPs and their active ingredients have to be approved and/or authorized prior to their placing on the market and use. The present chapter focuses on the regulatory toxicology aspects and requirements for agrochemicals.

The term "agrochemical" comprises any component of a PPP, e.g., active substances (ASs), synergists, safeners, solvents, solid carriers, or emulsifiers. This chapter deals mainly with the active substances, safeners, and synergists because they receive most of the toxicological attention stipulated by regulatory requirements. The non-active components, also called co-formulants, while not neglected, play a lesser role for the toxicological assessment of PPPs.

The present chapter mainly reflects the regulatory situation in the European Union, but the principles of agrochemical regulation are similar in many regions of the world.

Regulation of Agrochemicals

In most world regions, PPP registration follows a two-stage process. In the first stage, all active substances (and also all synergists and safeners, if relevant) in the PPP have to be approved. As soon as all active substances in a PPP are approved, the PPP itself needs to be authorized. The majority of toxicological data is generated for the approval of the active substance.

Data Requirements for Active Substances

The current data requirements for active substances are shown in Table 1.

The testing into **irritating and corrosive properties** should follow a tiered approach with the aim of avoiding in-vivo testing, especially when the AS is a strong irritant or corrosive. An in-vitro test for skin corrosivity should be conducted

Table 1 Toxicological data requirements for active substances in the European Union (Commission Regulation (EU) No 283/2013 of 1 March 2013)

Endpoint	Requirement
Absorption, distribution, metabolism, and excretion (ADME)	
ADME in rats, oral route	Required, single high dose, single low dose, and repeated low dose
Comparative in vitro metabolism study	Required, liver microsomes, human and any relevant species from pivotal studies
Acute toxicity	
Acute oral toxicity	Required
Acute dermal toxicity	Required
Acute inhalation toxicity	Required if vapor pressure $> 1 \times 10^{-2}$ Pa at 20 °C the AS is a powder containing >1% (w/w) particles < 50 μm the AS is included in PPPs that are powders or are applied by spraying
Primary eye irritation	Required, in vitro
Primary dermal irritation	Required, in vitro
Skin sensitization	Required, local lymph node assay (LLNA) preferred
Phototoxicity	Required if extinction coefficient is ≥10 L/mol/cm in the 290–700 nm range
Short-term toxicity	
Oral 28-day study	If available
Oral 90-day study	Required, rat and dog
Genotoxicity	
Bacterial assay for gene mutation	Required
Clastogenicity in mammalian cells, structural and numerical aberrations	Required Preferred: in vitro micronucleus assay
Bacterial assay for gene mutation	Required unless already positive from *hprt* or mouse lymphoma assay
In vivo studies in somatic cells	Required, in vivo micronucleus test
In vivo studies in germ cells	Normally not required
Long-term toxicity and carcinogenicity	
Long-term oral toxicity and carcinogenicity study (2 years) in the rat	Required, preferably combined
Carcinogenicity study in the mouse	Required
Reproductive toxicity	
Generational studies	Required. Two-generation study or extended one-generation study in rats
Developmental toxicity studies	Required, rat and rabbit
Neurotoxicity	
Neurotoxicity studies in rodents	Required for structural analogues to known neurotoxicants or if specific indications for neurotoxicity have been observed in other toxicity studies

(continued)

Table 1 (continued)

Endpoint	Requirement
Delayed polyneuropathy studies	These studies shall be performed for active substances of similar or related structures to those capable of inducing delayed polyneuropathy such as organophosphorus compounds
Other toxicological studies	
Toxicity studies of metabolites	Metabolites that are detected in plants or animal products, soil, or groundwater are tested to establish their relative hazard compared to the active substance if they are not major metabolites in the main test species (mostly the rat)
Supplementary studies on the active substance	These studies are mostly performed to elucidate the mechanism of action behind critical effects observed in the standard tests. Frequently, the human relevance of tumors observed in rodents is the subject of these studies

first, if this study is positive, the AS can be classified accordingly and no further testing for eye effects, dermal toxicity, or skin sensitization needs to be conducted.

Regarding **genotoxicity**, positive findings in any in-vitro test need to be followed up by a suitable in-vivo test. A positive clastogenicity assay has to be verified by an in-vivo micronucleus assay. However, this test is part of the basic data set. The situation is more complicated in case of a positive gene mutation test in bacteria or mammalian cells. This would require an in-vivo gene mutation assay as follow-up investigation. A transgenic rodent somatic and germ cell gene mutation assay are among the recommended tests to satisfy this data requirement. This regulatory use of this assay is still relatively new, and experience with this assay is not widespread, both among industry and regulators.

In addition to these basic data requirements, studies into **endocrine disrupting properties** may be triggered, depending on the quality of the database and on possible concerns raised by findings in the available data.

Data Requirements for Plant Protection Products

The current EU data requirements for PPPs are shown in Table 2. Vertebrate studies shall not be conducted if the toxicity for each endpoint can be reliably predicted using the calculation methods laid down in the CLP Regulation 1272/2008.

Human Health Risk Assessment

The human health risk assessment for agrochemicals follows the principle of comparing measured or estimated exposures to regulatory reference doses.

Table 2 Toxicological data requirements for plant protection products in the European Union (Commission Regulation (EU) No 284/2013 of 1 March 2013)

Endpoint	Requirement
Acute oral toxicity	Required
Acute dermal toxicity	Required
Acute inhalation toxicity	Required if the PPP (a) Is a gas or liquefied gas (b) Is a smoke-generating plant protection product or fumigant (c) Is used with fogging/misting equipment (d) Is a vapor-releasing plant protection product (e) Is supplied in an aerosol dispenser (f) Is in a form of a powder or granules containing >1% (w/w) particles <50 μm (g) Is to be applied from aircraft in cases where inhalation exposure is relevant (h) Contains an active substance with a vapor pressure $> 1 \times 10^{-2}$ Pa and is to be used in enclosed spaces such as warehouses or glasshouses (i) Is to be applied by spraying
Primary eye irritation	Required, in vitro
Primary dermal irritation	Required, in vitro
Skin sensitization	Required, LLNA preferred

Exposure Assessment

Dietary Exposure

Plant protection plays an essential role in production of agricultural crops. Assessment of exposure of consumers to pesticide residues in their diet or drinking water is therefore an important aspect in the regulatory process. Residues of the active substance(s) and their potential metabolites in food matrices of plant or animal origin are measured. Using these residue levels as input, acute and chronic exposures via diet can be estimated via food basket models such as PRIMo (EFSA 2019).

Pesticide residues in drinking water must not exceed the legal limit of 0.1 μg/L set by the EU Drinking Water Directive 98/83/EC. Residue levels in drinking water can be estimated using designated modeling algorithms, simulated application onto soil (lysimeter studies), or in rare cases, by actual sampling.

Non-Dietary Exposure

Exposure to ASs in PPPs is estimated for operators, workers, bystanders, and residents. Operators are individuals who apply PPPs by spraying, spreading of granules, seed treatment, etc. Operators may be exposed to undiluted PPP while mixing and loading application equipment and also to diluted spray broth during application. Workers are potentially exposed to residues of the PPP when re-entering treated cultures, e.g., for harvesting, manual weed control, or other operations.

Bystanders and residents are members of the general public who may stand, walk, or live in the vicinity of treated cultures. Bystanders can be exposed via inhalation or dermal contact with drifting spray mist. Residents, including children, could encounter residual PPP on their own lawn.

Exposure models are available for estimating exposures of the described subpopulations with a variety of application conditions. In the EU, the Agricultural Operator Exposure Model (AOEM) is currently the recommended exposure model that covers the most common application techniques and exposure situations (EFSA 2014).

Exposure models yield conservative estimates by their very nature. In cases where the modeled exposure estimates exceed the reference doses, actual exposure measurements can be undertaken. In such exposure studies, operators wearing dosimeters apply the PPP in question under field conditions. Similarly, dosimetry on mannequins simulating bystanders can be performed to refine the exposure estimation.

Non-dietary risk assessment is based on systemic reference doses (see following section "Hazard Assessment"). It is therefore necessary to account for the absorption of the AS via various routes of exposure. In most cases, only measured dermal and oral absorption data will be available, while 100% absorption will be assumed for the inhalation route. The extent of oral absorption is estimated based on the ADME studies with the AS itself. In contrast, dermal absorption studies are performed with the formulated PPP (as concentrate and in-use dilution) to account for matrix and dilution effects. In the absence of suitable dermal absorption data, default vales can be used, depending on the physical state of the PPP, whether it is water- or solvent-based and on whether the concentrated or diluted form of the PPP is assessed. The rules for evaluation of dermal absorption data and the application of default values are laid down in a specific guidance document by EFSA (EFSA 2017).

Hazard Assessment

The risk assessment is based on reference doses that are typically set using No-Observed-Adverse-Effect Levels (NOAELs) as points of departure. NOAELs are adjusted by division by an assessment factor that accounts for inter-species differences between experimental animals and humans as well as intraspecies variability among exposed populations. Acute, subchronic, and chronic NOAELs are considered, depending on the duration of the exposure scenario for the exposed population.

The acceptable operator exposure level (AOEL) is the relevant reference dose for all modes of nondietary exposure. Since PPP applications are mostly restricted to certain seasonal growth stages, AOELs are often derived from NOAELs of subchronic toxicity studies. For active substances with substantial acute toxicity, an acute AOEL (AAOEL) is also necessary. In current regulatory practice, the (A) AOEL is corrected for systemic bioavailability if the measured oral absorption is lower than 80%.

For dietary risk assessment, the acceptable daily intake (ADI) is the relevant reference dose covering chronic intake of the pesticide residues via food and

drinking water. The ADI is typically derived from the lowest long-term NOAEL. For active substances with appreciable acute toxicity, the acute reference dose (ARfD) is also derived. Since there are currently no designated test protocols for establishing acute NOAELs, the ARfD is often based on short-term studies such as acute neurotoxicity or developmental toxicity studies. Unlike the (A)AOEL, ADI and ARfD are not corrected for oral bioavailability.

If a PPP contains several ASs, a combined risk assessment for human health is required. In a first tier, dose additivity will be assumed, i.e., several ASs will affect the same target in an additive fashion. If the combined coverage of the respective reference dose exceeds 100%, a refined assessment will be necessary. This refinement requires an analysis of each of the effects caused by each single AS. If, for instance, several ASs in the PPP affect the liver at a certain dose, a reference dose (e.g., an AOEL) for the target organ "liver" will have to be considered. If the combination of organ-specific AOEL coverage still exceeds 100%, a further refinement might look at different liver cell types that are affected (e.g., bile duct epithelium versus parenchymal cells) or a detailed mode-of-action analysis could support the independent, nonadditive action of two or more ASs. It is obvious that the situation can get very complicated if this risk assessment is extended to residues and their metabolites in food, and regulatory guidance for such an assessment is not yet available on EU level.

In addition to the quantitative risk assessment relative to these reference doses, there are hazard-based exclusion criteria that will exclude the AS from being approved, unless human exposure to the AS or its residues is negligible. This may apply to PPPs used in closed systems. The exclusion criteria comprise ASs that are classified as carcinogenic, mutagenic, or toxic to reproduction (Category 1A or 1B; CMR) or that are considered to have endocrine-disrupting (ED) properties. ASs that are persistent, bioaccumulative, and toxic to the environment (PBT) are also excluded from approval.

Thus, ED properties will normally lead to non-approval of the AS. Only quite recently, a first guidance document for assessment of ED properties has been published (ECHA and EFSA 2018). This assessment focuses on ED properties mediated via estrogenic, androgenic, thyroid hormone, or steroidogenic (EATS) pathways.

The OECD-testing guidelines in their current versions include hormone measurements (OECD TGs 408, 414) or assessment of sexual maturation in newborn animals (OECD TG 416). If an AS has been tested according to the latest versions of the test guidelines, the absence of ED-mediated adverse effect allows the conclusion that the AS is not an ED substance with relevance for human health.

However, this is currently an unlikely scenario as most active substances on the market have been tested according to earlier guideline versions that lack the ED-relevant parameters. Thus, a weight-of-evidence assessment of the available data needs to be conducted. It is likely that at least in-vitro screening assays into effects on estrogen- or androgen-receptor activation and steroidogenesis need to be performed for most existing ASs.

In addition to ED effects with human relevance, an assessment of the ED potential with regard to the environment is also conducted, but this is outside the scope of this chapter.

Authorization of Plant Protection Products

In the EU, "authorisation of a plant protection product" means an administrative act by which the competent authority of an EU Member State authorizes the placing on the market of a PPP in its territory.

A PPP can only be authorized if all the ASs (and any synergists and safeners) it contains have been approved.

The PPP is subjected to a human health risk assessment as described above, based on the reference doses set for all ASs in the product under the use conditions proposed by the applicant. The toxicological data set for the PPP mainly serves classification and labeling and ensures that appropriate protective measures are taken when handling the PPP.

Assessment of efficacy and environmental safety are also essential cornerstones for the successful authorization of a PPP.

Approval of Active Substances

In the EU, an AS is evaluated by a member state competent authority, acting as so-called "rapporteur Member State" (RMS) on behalf of the EU member states. A co-RMS is assigned to assist with the evaluation.

The producer of the AS submits to the RMS an application and a dossier containing the information stipulated by Regulation 1107/1009. The RMS should prepare a Draft Assessment Report (DAR) within 12 months after the dossier has been found to be complete. This 12-month period can be interrupted by so-called "stop-the-clock" procedures when the RMS requires the submission of additional information that is found necessary in the course of the evaluation process.

The DAR is then commented on by other MS, and the European Food Safety Authority (EFSA) will organize a peer review of the DAR. EFSA will draw a conclusion on the evaluation and recommend the approval or nonapproval of the AS and any conditions for the approval. Approval will typically be granted if at least one safe use for the AS could be demonstrated. This use is assessed based on information on a representative product which is assessed for its safety to humans and the environment. In addition, the hazard-based exclusion criteria (CMR, ED) must not be met.

The approval act is completed by an Approval Regulation issued by the EU Commission. The first approval of an active substance will be for a maximum period of 10 years. The renewal of an approval can be granted for a maximum of 15 years. To allow a seamless transition between approval periods, the renewal application must be submitted no later than 3 years before the expiry of the approval.

The Glyphosate Controversy

The approval procedure and criteria for active substances are laid down in Annex II to Regulation 1107/2009. The procedure is the evaluation of scientific data by member states and EFSA experts.

However, the example of the glyphosate approval renewal shows that political pressure can supersede the recommendations of the scientific experts. The German competent authority as rapporteur proposed a renewed approval for 15 years in the renewal assessment report dated 18 December 2013. In 2015, the International Agency for Research on Cancer (IARC) published a monograph in which glyphosate was classified as "probably carcinogenic to humans" (Group 2A; IARC 2015). This prompted the EU Commission to mandate a reassessment by EFSA of the carcinogenicity data for glyphosate. The reassessment confirmed the initial finding that glyphosate is not carcinogenic. The subsequent substance evaluation by the European Chemicals Agency (ECHA) also confirmed that glyphosate is not carcinogenic (ECHA 2017). The difference between EU and IARC assessments can at least partly be explained by the fact that IARC did not take the numerous regulatory studies in which no carcinogenic activity was observed into account (see Greim et al. 2015 for review). IARC claimed that the full study reports were not available for assessment. Instead, epidemiological studies in which exposure to glyphosate-containing formulations was identified as risk factor for tumor diagnoses were weighted high in the IARC assessment, even if none of the critical studies were scientifically robust (Acquavella et al. 2016).

However, the IARC evaluation had already sparked initiatives by non-governmental organizations toward an EU-wide ban of glyphosate, and a European Citizens' Initiative garnered enough signatures so that the EU Commission had to consider the proposal for a ban. Member states could not agree on either approval or non-approval, and the decision was postponed. Finally, a renewed approval for 5 years was agreed on by member states. Thus, the initial 15-year approval proposal was turned into a 5-year renewal, even though all concerned scientific bodies in the EU agreed that glyphosate was non-carcinogenic.

The example of glyphosate approval in the EU goes to show that the political decisions can overrule scientific judgment.

Co-Formulants

Co-formulants are non-active ingredients of a PPP that do not qualify as safeners or synergists. Co-formulants comprise, e.g., solvents, thickeners, surfactants, emulsifiers, or pigments. The use of co-formulants is restricted by Annex III to Regulation 1107/1009 which is essentially a negative list. Substances listed on Annex III are not accepted for inclusion in a PPP.

Co-formulants essentially underlie the same exclusion criteria as ASs. With regard to human health effects, co-formulants must not be classified as carcinogenic, mutagenic, or toxic to reproduction (Category 1A or 1B) or have ED properties.

An import example of a now-banned co-formulant is the polyethoxylated (POE) tallow amines (CAS No. 61791-26-2) which were frequently used in glyphosate-based herbicides. Even though the polyethoxylated tallow amines did not fulfil any of the exclusion criteria (e.g., CMR, ED, and PBT), EFSA concluded that "compared to glyphosate, a higher toxicity of the POE-tallow amine was observed on all endpoints investigated." Additional concerns were highlighted as regarding the potential of POE-tallow amine to negatively affect human health, and POE tallow amines were excluded from use in PPPs containing glyphosate. The ban was extended to all PPPs by the EU Commission, and POE tallow amines were added to Annex III of Regulation (EC) No 1107/2009.

There is no data requirements for co-formulants under Regulation (EC) No 1107/2009. It suffices to submit safety data sheets for co-formulants along with the PPP authorization dossier. However, co-formulants must be registered for their use in PPPs under the REACH Regulation 1907/2006. The use pattern of agrochemicals differs from the professional or industrial use of other chemicals, e.g., the application mostly occurs in the open field, resulting in intentional release into the environment. The standard tools for human and environmental exposure assessment that are regularly employed for industrial uses are not suitable for agricultural applications. To facilitate the risk assessment for co-formulants under REACH, the European Crop Protection Association (ECPA) has developed the "REACH-IN" initiative. REACH-IN provides risk assessment tools fit for agrochemical applications that also meet the requirements of REACH (Dobe et al. 2016; Mostert et al. 2019). The co-formulant uses should preferably be registered by the manufacturer or importer of the coformulant. However, niche uses as in PPPs may be easily overlooked by manufacturers of bulk chemicals. In this case, the agrochemical use may also be registered by the PPP manufacturer in a downstream-user chemical-safety report.

Cross-References

- ▶ Examination of Acute and Repeated-Dose Toxicity
- ▶ Limit Values and Guideline Values in Regulatory Toxicology
- ▶ Registration and Approval in Regulatory Toxicology
- ▶ Single Substances Versus Combined Effects: Problems in Mixture Toxicology
- ▶ Toxicological Risk Assessment in Different Jurisdictions

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Biopharmaceuticals: CMC Development "Points to Consider" from a Regulatory Perspective

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Mike DiPaolo and Mathias Locher

Contents

Abstract

Small molecule therapeutics are chemically defined compounds. Their large-scale production and the molecular structure are highly defined. In contrast, biopharmaceuticals are large and very complex molecules and described by its primary, secondary, tertiary, and quaternary structure. To make it more complex, biopharmaceuticals are recombinantly produced in cells by transfecting the cells

M. DiPaolo (✉)
BioTherapeutic Development and Supply, Janssen Research and Development, Malvern, PA, USA
e-mail: MDipaolo@its.jnj.com

M. Locher
Locher Biotech Consulting GmbH, Kuessnacht am Rigi, Switzerland
e-mail: mlocher56@icloud.com

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with the genetic code of the protein. In the early days, different cell types are used for protein production, but today Chinese hamster ovary cells (CHO cells) are the cells used for biopharmaceutical production. In this chapter, all the important aspects for biopharmaceutical production like cell line development, cell clone selection, upstream development (USP), and downstream development (DSP) will be discussed.

Keywords

IMPD · Pharmaceutical · Drug · Product quality · GMP manufacturing · Master Cell Bank (MCB) · Stability testing · Protein characterization · Comparability · Analytical testing

Introduction

The global therapeutic protein market was valued at about $93.14 billion in 2018 and is expected to grow to $172.87 billion at a compound annual growth rate (CAGR) of 16.7% through 2022 (Therapeutic Proteins Global Market Report 2020). In order to benefit from this growing market, it is imperative that those involved in the chemistry, manufacturing, and control (CMC) aspects of biopharmaceutical products provide "first time right" product development information in the quality module of the common technical document (CTD) to health authorities. By doing so, sponsors can expect to obtain approval to conduct clinical trials and ultimately to obtain marketing authorization for their drug candidates in the most expeditious time possible. Not knowing the expectations of the health authorities who review the CMC information in regulatory filings can cause delays in the approval process and therefore delays in obtaining marketing approval for the sponsor's drug and associated loss of revenues. In the following paragraphs of this chapter, the author will review and discuss "CMC points to consider" from a regulatory perspective when developing a protein-based therapeutic. These points to consider are not as much from any one health authority's "points to consider" or "code of regulations" but are a culmination of experiences from the author's 35+ years in drug development and contributions to regulatory filings and responses submitted to health authorities. While health authorities may be consistent generally on the majority of drug development requirements and topics, it is highly likely that at least one health authority may have a different standard or perspective on a given topic in a global filing. The author also does not claim that this list as "all inclusive." The information discussed is some of the more critical aspects and requirements for biotechnology and biopharmaceutical companies to consider when developing the filing strategy for an investigatory new drug (IND) application or Investigational Medicinal Product Dossier (**IMPD**). The authors recommend that the readers review the guidance documents listed for more information. In the following chapter, the author defines the term "new molecular entity" or "NME" as a generic term applied to a new therapeutic molecule that enters formal drug development at the preclinical toxicology stage.

Foundations of Drug Development: TPP, Quality TPP, CQAs, and Control Strategy

In order to stress the importance of developing a drug candidate in a pragmatic manner, FDA published a draft guidance document on the concept of target product profile (TPP) and its role guiding the overall drug development strategy for a clinical drug candidate (Guidance for Industry and Review Staff 2007; Part 1 2008; Target product). The TPP is a format to summarize the drug development program of a new molecular entity (NME) as described in terms of the drug's intended label at commercialization. The TPP describes, at a high level, the goals of the drug development program and documents the studies that are required to support the label in a manner that allows for future interactions with the FDA. Key sections of the TPP from a CMC perspective which become the quality TPP (QTPP) are as follows:

- Dosage and administration
- Dosage forms and strengths
- How supplied/storage and handling

To drill deeper into the requirements of the TPP, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) issued Q8 which describes in greater detail the concept of the quality TPP (QTPP). The QTPP provides an understanding of what will ensure the quality, safety, and efficacy of a specific product for the patient. In the ICH quality guideline ICH Q8(R2), guidance is provided on the goals for pharmaceutical development in the overall drug development strategy or QTPP. Sponsors should define the quality target product profile (QTPP) of an NME and include attributes such as the route of administration, dosage form, bioavailability, strength, and stability. Quality attributes of an NME are those that directly impact quality, safety, and efficacy. In addition, ICH Q8 provides guidance on identifying potential critical quality attributes (CQA) and then defining a control strategy for those attributes to ensure that a product is consistently manufactured to meet those attributes. The QTPP therefore describes the design criteria for the product and should form the basis for the development of CQAs, critical process parameters (CPP), critical attributes (CMA), and the overarching control strategy.

ICH Q8 defines CQAs as a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality. These are attributes of the molecule that the sponsor needs to control to ensure that a product is safe and efficacious. Some of the CQAs will originate from health authority expectations and others from the molecule's mechanism of action. For example, an antibody which binds to a target cancer cell and then elicits an ADCC response to kill the target cancer cell would have both antigen binding and Fc effector function activity as CQAs. In addition, it is known that the fucosylation levels of an antibody designed to elicit an ADCC response are also critical to achieve the desired effect (i.e., low fucose levels

correlate to higher ADCC activity). As such, fucose levels would also be a CQA for such an antibody. In addition to those CQAs that are related to the MoA, CQAs related to purity (% monomer), strength (protein concentration), quality (sterility, endotoxin content), identity, and potency (bioactivity) are also expected CQAs for a therapeutic protein. A final subset of CQAs fulfilling health authority expectations such as color, turbidity, pH, and appearance also need to be identified as CQAs and controlled by the sponsor.

"Control" of a CQA can be achieved by (1) controlling the raw materials needed to manufacture the product, (2) in process and final product testing, or (3) controlling critical manufacturing process parameters (CPPs). A CPP is a process parameter whose variability has an impact on a CQA and therefore needs to be monitored or controlled to ensure that the process produces the desired quality product. Examples of CPPs include the production bioreactor temperature and pH set point and allowable range and the duration that cells are grown (production day). Although not mentioned in ICH Q8, the concept of control strategy is also applied in the biopharmaceutical industry to the quality of raw materials used to manufacture the product. It would therefore also be critical to define the critical material attribute (CMA) of all raw materials used in the manufacturing process to ensure that a safe and efficacious product is consistently manufactured. A CMA is a physical, chemical, biological, or microbiological property or characteristic of an input material that should be within an appropriate limit, range, or distribution to ensure the desired quality of the output material. Sponsors therefore also need to define the CMAs as part of their control strategy.

In summary, health authorities expect sponsors to define the QTPP, control strategy, and associated CQAs, CPPs, and CMAs in great detail in a market application. In order to gain approval, the sponsor will need to provide assurances (i.e., drug development data) in the marketing application to demonstrate they have gained sufficient knowledge about the raw materials used in manufacturing and about the processes used to manufacture the desired commercial that consistently meets the defined specification. In essence, the specifications define the acceptance criteria for the product CQAs that are filed in the marketing application.

For more information on the concept of QTPP, control strategy, CQAs, CPPs, and CMAs, the author recommends that the reader review "A-Mab: A Case Study in Bioprocess Development" (A-Mab, 2009).

Quality Levels for Development and Manufacture of GLP, Phase 1/Phase 2, and Phase 3/Commercial Supplies

The health authority community develops and issues regulations and guidances to protect humans enrolled in clinical trials and in the commercial marketplace. These documents do not apply when specifying the quality requirements needed to manufacture GLP toxicology supplies. The regulations do specify that such supplies do not need to be manufactured under the same (i.e., GMP) conditions

necessary to produce drugs for clinical evaluation; however, supplies used in GLP preclinical safety studies do need to be representative of those to be subsequently used in clinical trials. Regulations do require that the testing of GLP supplies be performed using qualified methods under the auspices of an overarching quality system (i.e., quality assurance oversight). The reason for this is that the GLP and GMP regulations that focus on testing serve two different purposes. GLPs are designed to protect scientific data integrity and to provide health authorities with a clear and auditable record of open-ended research studies. In contrast, GMPs are intended to demonstrate to the health authorities whether or not individual batches of a regulated product are manufactured according to predefined manufacturing criteria. Given the time constraints and sense of urgency that many drug developers work under, it is best practice to use the same qualified methods to test the first in human (FIH) enabling GLP supplies and P1 supplies. The reason for this is the data generated on both test articles can be used to support comparability of GLP supplies to P1 supplies as this exercise is necessary if the sponsor manufactures GLP supplies and P1 supplies under different conditions such as scale or facility location. Additionally, since the products are tested by the same methods, the sponsor will also be able to use the stability data on the GLP toxicology batch to set the expiration date for P1 supplies which are typically manufactured at a later time.

Analytical Development

Analytical methods should be validated for their intended use in early development. A method validation study needs to be performed under an approved protocol for each method used for in-process and release/stability testing. The protocol needs to specify the procedure with acceptance criteria for each method with respect to the method's specificity, accuracy, repeatability, precision, linearity, range, and limit of detection/quantitation. In early development, a validation study performed by one or two analysts on a single instrument may be sufficient. The sponsor typically describes each method used for in-process, characterization, release, and stability testing in Section 3.2.S.2.4 and 3.2.P.5.2 of the quality module in a CTA (clinical trial application) in an early development regulatory filing. The sponsor also provides a summary of the acceptance criteria and results from each method validation study for health authority review.

When a CMC program transitions to P3 late development, analytical methods need to meet the expectations for commercial methods as these methods will be used to test P3, validation, and commercial batches. P3/commercial methods need to be fully validated. A full validation will typically include multiple analysts using multiple instruments in both the analytical development lab and the commercial testing site. The sponsor will describe the commercial analytical methods and method validation results in much greater detail in the same sections as an early development filing in their commercial marketing application.

Forced Degradation Studies

Formulation scientists do their best to deliver a stable drug product formulation into the clinic. They perform screening studies to determine the optimal pH at which to minimize product degradation. In addition, they may add antioxidants (methionine, EDTA), stabilizers (sucrose, sorbitol), and surfactants as excipients to minimize/prevent the product from degrading over time in the final formulation buffer. Despite their best efforts, the product may inevitably degrade due to adverse environmental conditions such as temperature excursions and excessive light exposure during storage, excessive shaking during shipping, and time in solution. As such, biopharmaceutical companies should determine as soon as feasible what the potential degradation pathways and resulting products of their therapeutic protein are that will be under clinical evaluation. Sponsors should first perform a preliminary assessment by examining the amino acid structure of the NME to identify concerning "hot spots." Hot spots include amino acid sequences susceptible to clipping (e.g., ASP/PRO sequences), deamidation (such as ASN or ASP), or oxidation (MET or TRP). Without this data, the sponsor will not have sufficient insight to identify/determine which analytical methods need to be developed and implemented to assess changes in the critical quality attributes of the molecule at release and on stability. Degradation products can lead to increased toxicity/immunogenicity and/or in a reduction in the potency of the molecule or half-life. Therefore, health authorities expect sponsors to file the results from forced degradation studies in the regulatory filings and expect the sponsor to employ a sufficient number of analytical methods to detect changes in those degradant levels over time. Clipping whether it be by proteases or peptide bond hydrolysis under acidic conditions at susceptible amino acid sequences leads to fragmentation which can be monitored using analytical methods based on molecule weight such as size exclusion HPLC or capillary electrophoresis methods run under reduced conditions. Deamidation and oxidation species can be detected and tracked using charge-based methods such as capillary electrophoresis (cIEF) or ion exchange chromatography (IE-HPLC). Molecular changes can also be identified and quantified using peptide mapping techniques, but such methods are difficult to implement on a routine basis in a QC lab environment. Glycosylation is one of the most common posttranslational modifications (PTMs) of biopharmaceutical drugs and may have a pivotal role in their safety and efficacy by modulating a wide range of drug properties, including immunogenicity, in vivo circulatory half-life, and effector functions (13). Glycosylation levels typically do not change over time unless there are enzymes present in the product. The author has not seen this phenomenon during his tenure in drug development.

Stability

One of the biggest challenges facing development scientists is providing a stable drug product formulation in the appropriate presentation for initial clinical studies. Typically, the dose range for the P1 study is not known and may not be finalized until

after health authority feedback. As such, the required design space for a P1 formulation strength may need to span several log units. This is especially true on oncology programs where the starting dose could be as low as nanograms per kilogram body weight due to safety concerns with the mode of interaction (e.g., CD3 redirectors). The highest dose in the dose escalation scheme may reach mg/kg levels. For a single drug product formulation/presentation, to span the dosing requirements for the entire 5-log-unit range is challenging to say the least. The uncertainty posed by an unknown dose range to bracket translates into the development risk that a suboptimal concentration for active in the drug product is selected. If this occurs, it could negatively impact the stability of the drug product becoming a significant risk that the clinical study could be interrupted.

It is of the upmost importance that the formulation scientists base their formulation decision on a well-planned stability program to generate sufficient stability data with the lead formulation in the selected container closure system. Once the sponsor identifies the proper formulation and methods to assess the stability of the drug substance and product, it is time to develop a stability strategy. ICH guidelines are the most appropriate to follow. Based on the risk that the product will not be stable over the intended shelf life, the sponsor needs to demonstrate that they have enough knowledge about the stability of the drug product in the selected formulation in the regulatory filing to gain approval to start clinical trials. A typical strategy would be for the formulation scientist to first generate development data on several formulations. Many companies now use high-throughput screening in microtiter plates to evaluate multiple formulations in a short period of time. Once an acceptable lead candidate is identified, the formulator typically prepares a lead lot representative of the P1 clinical material. The resulting drug substance is filled as a lead lot in the P1 container closure system under comparable conditions at least 6–9 mo. prior to a regulatory filing. The clinical lot is subsequently filled 4–6 mo. prior to the filing. It is worth noting that the clinical drug product will lose at least 6 mo. shelf life prior to the administration of the first dose. By following this strategy, the sponsor will be able to file with 3–6 mo. data on the lead lot and 1–3 mo. data on the clinical lot.

The lead and first clinical lots are placed in a stability program under real-time, accelerated, and stressed conditions. Storage temperatures and testing time points for the stability program should be based on the International Conference on Harmonisation (ICH) guidelines (ICH Guideline Q1A Stability Testing of New Drug Substances and Products; ICH Guideline Q5C: Quality of Biotechnological Products, Stability Testing of Biotechnological/Biological Products). Table 1 is an illustration of a stability plan for a typical therapeutic protein drug product stored under refrigerated conditions.

By generating stability data in excess of the recommended storage temperature of 5 °C in a standard stability study, sponsors will be able to support temperature excursions that may occur when the product is stored in the warehouse, when the product is in transit to the clinical site, or when the product is stored at the clinical site. With acceptable accelerated and stressed stability data, the sponsor will be able to extrapolate the shelf life of the product from the real-time value. In other words, with acceptable 3 mo. stability data at all three temperatures (real time, accelerated,

Table 1 Generic stability monitoring program for a refrigerated drug product

					Month				
Test method	0[a]	1	3	6	9	12	18	24	36
Color of solution									
pH	A	C	ABC	AB	AB	AB	AB	AB	A
Turbidity	A	C	ABC	AB	AB	AB	AB	AB	A
Particulate matter (visible translucent)	A	C	ABC	AB	AB	AB	AB	AB	A
Particulate matter (subvisible)	A	C	ABC	AB	AB	AB	AB	AB	A
Protein concentration by A280	A	C	ABC	AB	AB	AB	AB	AB	A
Binding assay	A	C	ABC	AB	AB	AB	AB	AB	A
cIEF	A	C	ABC	AB	AB	AB	AB	AB	A
SE-HPLC	A	C	ABC	AB	AB	AB	AB	AB	A
cSDS (reduced)	A	C	ABC	AB	AB	AB	AB	AB	A
cSDS (non-reduced)	A	C	ABC	AB	AB	AB	AB	AB	A
Critical excipient level	A	C	ABC	AB	AB	AB	AB	AB	A
Sterility	A					A		A	A

a: Release data is T = 0
A = 5 °C, B = 25 °C, C = 40 °C

and stressed), the sponsor will be able to claim 6 mo. shelf life on the clinical supplies.

If the sponsor intends to change formulations during later stages of development, then there is limited information that can be gained studying additional batches of P1 clinical drug substance or drug product on stability. Under these circumstances, drug developers can save resources by not formally testing drug substance or drug product batches after the first clinical batch. The developer may choose to place subsequent batches in the appropriate stability chambers (i.e., place "on station") and then pull and freeze samples at – 80 °C at the designated time points. If there are inconsistencies in the stability data from the lead or first clinical lot, the drug developer can thaw the samples that were placed "on station" to generate additional stability data in order to make decisions about the stability of the molecule in the designated formulation.

In addition to the health authority expectation that the storage temperature and shelf life for drug substance and drug product be defined and supported, health authorities (especially in Europe) expect intermediate hold times to be justified by supportive data even in a P1 filing. Sponsors should generate stability data for all process step intermediates in their process development studies. When generating intermediate hold time data, a worst-case approach should be considered. For example, if the product is permitted to be stored at room temperature for 24 h, the sponsor should generate data at the highest temperature defined as room temperature (typically 15–25 °C so study at 25 °C) for a period of time in excess of >24 h. This will provide additional margin in case the manufacturing plant experiences a delay or a temperature excursion due to any downtime with the facility HVAC heating/cooling system.

Comparability

Health authorities consider the process used to manufacture a protein-based therapeutic product starting from clone selection to the final filled drug product as the investigational product. Any change to the cell line or the process to manufacture the biologic-based therapeutic could impact the structure of the molecule and hence the safety and/or efficacy of the molecule. Process changes include cell line changes, purification step changes, conjugation reaction step changes, manufacturing site/scale changes, and drug product presentation changes (e.g., liquid vial to lyophilized vial), to name a few. The more changes made to the "process," the more extensive the comparability exercise is required to demonstrate that the molecule pre- and post-change is highly similar/comparable. The key question to address from a regulatory perspective is: "Have any of the process changes caused the critical quality attributes of the molecule to be altered, and if so, what is the impact of those changes on product safety/immunogenicity and/or efficacy?" Changes that impact attributes such as aggregate or fragment levels can enhance the immunogenicity of the molecule and hence negatively impact the safety profile of the molecule. This would call into question the nonclinical and/or clinical study data previously generated with the pre-change product. As such, health authorities may insist that a safety study be conducted on the post-change molecule to ensure that there is no change on the safety profile of the drug. Changes to glycosylation patterns can cause changes with the FcRn binding characteristics and therefore impact the pK/half-life/Cmax. of the molecule (Zhang et al., 2016). If so, health authorities may require a pK study with the post-change molecule to ensure similar exposure levels pre- and post-change. The most impactful comparability requirement could be enforced if a process change(s) impact critical quality attributes associated with the activity of the molecule such as ability to bind to the target and elicit the necessary biological response. It is well known that fucose levels impact an IgG1's effector function activity. Low fucose levels are associated with higher effector function activity (such as antibody-dependent cell cytotoxicity or ADCC) as compared to higher fucose levels. Any change to cell line or media can impact glycosylation and therefore can impact biological activity. If these changes are significant, health authorities may require a study to assess the impact on efficacy if not also safety. Sponsors should consult with health authorities in advance on this topic. It is generally not "value added" to discuss comparability plans without having side-by-side pre- and post-change attribute testing data to propose a plan to address what the impact of any changes in attributes could mean on safety and efficacy.

Early Development Comparability Strategy

Realistically, there is minimal development time allotted to biopharmaceutical developers to define the process and formulation for GLP toxicology supplies. Sponsors want to find out as soon as possible with the minimal level of investment if an NME will progress into clinical development and then to the commercial market. GLP supplies are typically manufactured in a non-GMP facility at a smaller scale than P1 clinical supplies. They may also be manufactured with a

crudely developed purification process and formulated in a suboptimal excipient matrix. As such, any change in process, formulation, site, or scale will trigger a comparability exercise. If the change is relatively minor (a change in a column process step, change from a frozen liquid vial presentation to a refrigerated liquid or lyophilized vial presentation, change in manufacturing site, etc.), "CMC" comparability is generally sufficient. CMC comparability involves a detailed comparison of product quality attributes leveraging both release tests and characterization tests. Characterization tests must include those tests that can discriminate for changes in biological (Fc effector receptor binding or function, FcRn binding, target antigen binding, etc.), biochemical (carbohydrate analysis, amino acid sequence/modifications, etc.), and biophysical (sedimentation rate by ultracentrifugation, melting point, etc.) attribute differences. These tests should also be performed on stressed and stability samples to ensure that the molecule degrades at a similar rate yielding similar degradation products pre- and post-change(s). If changes, no matter how small, cause changes to critical quality attributes of the molecule, then in vivo comparability will be required to advance the asset into the next phase of clinical development.

Late Development Comparability Strategy

The goal of most biopharmaceutical companies is to have the commercial process implemented in the commercial manufacturing plant at commercial scale prior to manufacturing P2b pivotal or P3 clinical supplies. It is imperative that the clinical supplies used to generate the pivotal clinical study data be statistically representative (i.e., the "same") as commercial supplies for health authorities to approve a commercial license application. Product quality data generated on pivotal supplies is used to set specifications, and that data needs to be correlated to the clinical safety and efficacy data generated from the pivotal study. Given the large investment required to move into and support/conduct pivotal studies, biopharmaceutical companies will make most process changes required to ensure that the final process is robust and commercially viable from a cost of goods and capacity perspective, that the formulation is robust/stable, and that the presentation is focused on appeasing the patient prior to entering P3. Changes considered during P3 or prior to commercial launch should be highly scrutinized. Prior to P2b/P3, the sponsor may make cell line and media changes to boost titer and productivity, process changes to improve yield and throughput, and presentation changes (vial to prefilled syringe) to boost convenience and therefore market acceptance. Material representative of the P3 process and product should be manufactured as soon as possible so the data can be evaluated and compared to pre-change product data. Once changes are known, an acceptable comparability strategy as discussed previously can be reviewed with the health authorities. Health authorities may accept the proposal to approve process and formulation changes based on CMC comparability, or they may request that the changes/product attribute differences are significant enough to warrant that a clinical comparability study be conducted. If so, the sponsor will need to generate clinical data (pK, safety/immunogenicity, and/or efficacy) to support the differences seen in the post-change molecule.

Product Characterization: Elucidation of Structure and Impurities

The health authorities expect the sponsor to know their molecule and the impurities associated with their molecule so they can make an assessment about the safety of the molecule and the potential for toxicity associated with any impurities of the molecule. For the desired product and product-related substances, the sponsor should provide detailed information on the biochemical, biophysical, and biological properties of the molecule. The sponsor should demonstrate how the structure of the molecule determines the biological activity of the native protein. Biopharmaceutical companies employ a number of analytical tools to determine the primary, secondary, tertiary, and quaternary structure of the molecule in the hopes of linking the structure to the functionality/biological activity of the molecule. Examples of the analytical tools employed to determine the structure of a biotherapeutic are listed in Table 2.

Once the structure of the molecule is defined, the sponsor characterizes the biological activity of the molecule linked to the defined structure. Examples of the analytical tools employed to determine the functionality/biological activity of a therapeutic protein are listed in Table 3.

In addition to characterizing the product of interest, the sponsor must also identify and characterize product-related and process-related impurities associated with their molecule, typically those exceeding 1% relative abundance. Product-related impurities include aggregates and fragements of the native molecule and posttranslational modifications such as glycation, oxidation, deamindation, C-terminal lysine cleavage, pyroglutamate, etc. Process-related impurities include any chemicals used in the manufacture that could be carried through the process and present themselves in the final product such as protein A ligand, antifoam, or reduction agents. Impurities associated with the biological process such as host cell protein or DNA are also quantified and reported.

The sponsor is expected to provide the raw data (chromatograms, thermograms, mass spectums, curves, etc.) associated with those analyses used to elucidate the

Table 2 Analytical tools used for product characterization

Structure	Analytical tool
Amino acid sequence (primary structure)	Peptide map
Carbohydrate structure	Oligosaccharide map
Disulfide bond structure/free thiols	Non-reduce peptide map and Ellman's assay
Intact mass and mass heterogeneity	Mass spectroscopy
Charge heterogeneity	cIEF or ion exchange HPLC
Size heterogeneity	SEC-HPLC, SDS-PAGE, or cSDS
Secondary and tertiary structure	Circular dichroism spectroscopy, differential scanning calorimetry (DSC)
Quaternary structure	Sedimentation velocity analytical ultracentrifugation

Table 3 Analytical tools used to assess product functionality

Biological activity	Analytical tool
Target affinity	Target (receptor or antigen) binding or blocking assay
Biological activity	Cell-based assay which measures a downstream affect after binding is achieved or blocked
Binding to FcRn (neonatal receptor)[a]	TR-FRET
Binding to FcgammaR receptors[b]	TR-FRET
Charge heterogeneity	cIEF or ion exchange HPLC
Size heterogeneity	SEC-HPLC, SDS-PAGE, or cSDS
Secondary and tertiary structure	Circular dichroism spectroscopy
Quaternary structure	Sedimentation velocity analytical ultracentrifugation

[a]Expected of therapuetic antibodies
[b]Expected of therapeutic antibodies (e.g., IgG1) with effector functionality

structure and characterize the product and assocated product-related impurities in Section 3.2.S.3.1 of their regulatory filing.

Manufacturing Material Inputs

Master and Working Cell Banks

Master and working cell banks used to manufacture a biotherapeutic protein should be considered as the GMP starting material and therefore need to be manufactured under GMP with the origin of the host cell line and resulting clones fully traceable. The sponsor should describe in detail the origin of the production host cell line in 3.2.S.3.2.3 of the quality module. If the sponsor intends to use the host cell line for multiple NMEs, the sponsor should create a master and working cell bank prior to transfection and have the cell bank tested with the appropriate methods including safety.

Now that the host cell line has been properly documented to ensure traceability and quality, the sponsor would need to describe how the expression construct was created. A diagram depicting the expression plasmid with each gene called out needs to be illustrated in the dossier. The sponsor will then generate a number of clones containing the expression construct/plasmid and will need to select a single clone from that pool that will produce the molecule of interest at the desired level of quality and productivity. Multiple clones are screened for titer, stability, product quality, etc., at small scale. Possible lead candidates are selected and grown in small-scale reactors. The selection process continues until a single clone is chosen. The sponsor will then create a master and working cell bank and then extensively test those banks to ensure that they are free of bacterial, mycotic, and mycoplasma contaminants, as well as adventitious and endogenous viruses originating from the host cell line or

Table 4 Generic master and working cell bank testing plan

Tests	Testing plan	
	MCB	WCB
Viability	+	+
Purity (e.g., sterility, myco)	+	+
Retroviruses and other endogenous viruses (e.g., infectivity such as S+L-Focus Assay, reverse transcriptase)	+	–
Non-endogenous or adventitious viruses	+	+/–
Identity	+	+

+ = Test required
– = Test not required
+/– = Depends on test, not required if MCB was tested and not likely to change when creating WCB, e.g., QPCR for bovine viruses

any component used to make the cell bank. In order to determine what tests should be performed on the master cell, the sponsor needs to determine what the likely/potential sources of endogenous and adventitious agents are in the cell banking process. Potential sources include the host cell line and materials used to create or grow the cell line (e.g., fetal bovine serum), personnel involved creating the cell line/banks, and the environment the cell line/banks were created/manufactured. Once the sources are identified, a testing plan is developed as shown in Table 4.

The author recommends that the developer familiarize themselves with the cell bank testing requirements delineated in ICH Q5a and ICH Q5d for additional information on testing strategies.

The transfection process and the process to create the production cell line from research cell bank to master and/or working cell bank also need to be provided. The test results for the master and working production cell line also need to be provided to health authorities for their review in Sect 3.2.S.3.2.3. If animal-derived products such as fetal bovine serum were used in the cloning process or production cell line creation process, the sponsor must describe the sourcing of the material and any test results on the material and provide a Certificate of Suitability in that section as well.

Raw Materials

Health authorities expect the sponsor to control the quality of the raw materials used to manufacture their biopharmaceutical product. The controls implemented need to be described in the Control of Materials section of the dossier. For compendial raw materials, the sponsor will need to test those materials using the compendial methods and acceptance criteria of the countries where the product will be studied (i.e., in clinical trials) or markets launched/sold. If the product is intended for global studies or commercial marketing approval, then the sponsor would need to employ test methods and acceptance criteria that simultaneously meet USP/NF, EP, and JP standards. If the sponsor intends to conduct studies and launch in China, then ChP

standards must also be met. If the sponsor desires to market their product globally, it is best for the sponsor to meet all compendial requirements as early as possible but no later than at P3. If the sponsor reaches late development and finds out that one of the raw materials does not meet the standards for a region that they intend to commercialize in, it can pose a dilemma. The sponsor would either need to switch to a material that complies to the requirements for all markets which would require a supporting comparability program or the sponsor may choose not to commercialize in that region. The recently published ChP purity requirements for polysorbate are quite stringent, more so than other compendia. As a result, many companies are having to switch to alternative sources of polysorbate that differ from those used to manufacture their product. These companies now need to conduct comparability studies with their product formulated in polysorbate 80 sourced differently than what they have been manufacturing and clinical experience with if they wish to enter or remain on the market in China. If the product performs differently in the new polysorbate 80, the sponsor may not be able to conduct clinical studies in or market their product in China.

Not all raw materials used to manufacture a biopharmaceutical product are compendial. Cell culture media and chromatography resins fall into this category. For the non-compendial raw materials, health authorities expect the sponsor to establish and provide product specifications, certificates of analyses, and other information on the qualitative composition and controls of those materials. If those raw materials are considered proprietary by the manufacturer, the manufacturer will most likely not share the necessary product information with the sponsor. In those instances where the manufacturer has filed a Drug Master File, a letter of authorization (LOA) from the supplier should be provided in the dossier which authorizes the health authorities to view the supplied information on the composition of the material.

It is well known within the biopharmaceutical industry that all raw materials and components used in the manufacturing process be derived, whenever possible, from nonanimal sources. This requirement applies to media components (human recombinant insulin and transferrin vs. animal derived) and excipients (vegetable-derived vs. animal-derived polysorbate). What may not be as well known is that even reagents used in the subcloning process to create the manufacturing cell line are viewed as a "raw material" by health authorities. As such, the material needs to be certified as being animal-free.

In order to prove to the health authorities that the sponsor is in control of the raw materials used to manufacture their products, it is recommended that the following steps be proceduralized and implemented. The sponsor should develop a master certificate for every raw material established that can be provided to the health authorities upon request. Each master certificate should contain the following minimum documentation:

- Product specification and certificate of analysis for each batch received.
- For excipients, a GMP certificate and formal risk assessment as required per EMA directive 2015/C 95/02.

- Certificate to confirm the absence of human or animal components.
- BSE/TSE certificate and/or certificate of suitability (if appropriate).
- Melamine statement (if appropriate).
- For media and feeds, a completed questionnaire containing the qualitative composition of critical components should be requested from the manufacturer.

Taking these steps when the sponsor first develops their supplier and raw material qualification effort will save the sponsor time and effort on the back end during regulatory filing reviews or during quality audits.

Specifications

Health authorities expect the NME under consideration to be tested at key intermediate steps and at the final product stage and compared to a predefined acceptance criteria with limits or specifications. Tests performed depend on the critical quality attributes of the product and the defined control strategy for that attribute as described earlier in this chapter. The test employed for each attribute as well as the acceptance criteria are justified in 3.2.S.4.5, Justification of Specification of the dossier. Such testing is to be performed when the product is first manufactured (release or T = 0 testing) and at predefined times that the product is stored at predefined temperatures. Key intermediate steps are steps in the process where the product may be stored for extended periods at a predefined (and supported) temperature and time prior to further processing. The drug substance (i.e., formulated or preformulated bulk) stage is a typical intermediate storage step leveraged by companies prior to the drug product filing stage. As such, the drug substance is tested according to the specification. The final drug product be it formulated bulk in a vial or prefilled syringe is also tested at release and on stability. The release results are described in the certificate of analysis (CoA) which is shared with heath authorities in the regulatory filing.

Health authorities have been moving away from accepting acceptance criteria with "report results" and now expect all release tests to have acceptance criteria with numerical limits defined. For most therapeutic proteins, a specification for monomer of >/ = 95% is typically expected. With a monomer specification of >/ = 95%, impurity levels for higher molecular weight species or aggregate levels and low molecular weight species such as fragment would therefore need no more than 5% in total. Higher impurity levels than 5% would need to be justified by the sponsor and qualified as acceptable in the toxicity study.

In the commercial filing, health authorities expect the commercial specification to be based on a statistical analysis of the batches made and used in the clinic. As such, the sponsor needs to manufacture as many pivotal process batches as possible and include those batches (especially aged batches) in the pivotal trial. If possible, the sponsor should try to manufacture products with as much "variability" as possible so they can gain clinical experience with those batches and seek a commercial specification as broad as possible in the marketing application.

Table 5 Generic drug substance specification for an early development biopharmaceutical

Attribute	Test method	Acceptance criteria	
		Release	Stability
General characteristics	For example, pH, osmolality	Range A–B	Range A–B
Identity		Identity confirmed	N/A
Quantity	Protein concentration	C +/− 10%	C +/− 10%
Potency	Binding assay	D +/− 30–50%	D +/− 30–50%
Charge heterogeneity	cIEF or HPLC	Conforms to RRM Main peak : E–F% Sum of acidic : G–H% Sum of basic : I–J%	Conforms to RRM Main peak : E–F% Sum of acidic : G–H% Sum of basic : I–J%
Purity	By two orthogonal methods	Main component : >/= 95% Other species </ = 5%	Main component : >/ = 95% Other species </ = 5%
Process impurities	Residual protein A Residual HCP Residual DNA	</ = 50 ng/mg </ = 100–200 ng/mg <10 ng/dose	N/A N/A N/A
Microbial safety	Bioburden Endotoxin	</ = 10 CFU/mL K/M[a]	N/R N/R

N/A not applicable
N/R not required if container closure integrity is demonstrated
RRM research reference material
[a]K/M where K = 5 USP-EU/kg of body weight for any parenteral route of administration other than intrathecal and M = the maximum recommended bolus dose of drug per kg of body weight (Endotoxin Limits for Parenteral Drug Products 2017)

The specification for drug substance is described in S.4.1 and for drug product in Sect. P.5.1 Specifications. The specification is broken down into release specification (the acceptance criteria to release product at T = 0) and stability specification (the acceptance criteria which need to be met over the shelf life filed). A generic drug substance specification for an early development biopharmaceutical is provided in Table 5.

The reader is encouraged to familiarize themselves with ICH Q 6a (ICH Topic Q 6A Specifications) for more information on how to determine the necessary analytical methods required to establish specifications for their drug substance and drug product.

Compatibility

The sponsor not only needs to control the quality of the product from the creation of the production clone through the manufacture and storage of the labeled drug product steps, they also need to document control during the preparation and

administration of the drug product to the patient. The sponsor not only needs to define the procedure to store, prepare, and administer the drug, they also need to test those steps to ensure the quality of the product is still assured. In order to do so, the sponsor will conduct simulation studies mimicking the same drug product storage, preparation, and administration steps through the completion of the dosing process at the clinical sites, hospital/clinic, or patient's home. In these studies, the sponsor will define the acceptable time limits (e.g., 8 h) to prepare and administer the drug under the permitted environmental conditions (e.g., room temperature, protected from or under light). Depending on the diluent and the country, if the preparation and administration time is expected to exceed 4 or 8 h, the sponsor will need to conduct microbial challenge studies to ensure that the risk of contamination is properly mitigated by the preparation and administration procedure. The sponsor will define exactly how to prepare the drug if dilution is required and what the acceptable diluent(s) is/are. The sponsor also needs to define the acceptable material of construction (e.g., polypropylene, polyvinyl chloride, etc.) and the diluent if used (e.g., saline) for the ancillary supplies such as IV bags, administration sets, and syringes that are needed to prepare and administer the drug. Results from simulation testing need to affirm that the product attributes monitored in the study are not altered by the handling, preparation, and administration process and that the intended dose (quantitatively) is safely administered to the patient. A summary of the compatibility testing strategy and the results from the simulation study are provided to the health authority reviewer in Sect 3.2.P.2.6: Compatibility of the dossier.

Viral Clearance

Many biopharmaceutical products are produced by mammalian cell culture-based processes. The mammalian cell lines leveraged include Chinese hamster ovary and SP20 cells. Even though CHO cells have never been found to harbor infectious rubella virus-like particles, RVLP (although they always show type A and sometimes type C RVLP when tested and have always been found to be noninfectious in 40 years of testing by numerous labs), for purposes of risk assessment and viral clearance, CHO particles are assumed to be infectious. As such, these "potentially" infectious particles need to be accounted for and removed from the process to a "safe" level. Most biopharmaceutical companies follow the following strategy to demonstrate adequate levels of viral clearance by process steps that are after the production bioreactor step to achieve a theoretically acceptable level of virus particles per dose.

First, the number of potential infectious particles is assessed by performing analyses on the end-of-production cells from the production bioreactor stage. End-of-production cells are analyzed by TEM (transmission electron microscopy) and the number of particles quantified and the number of infectious particles per mg of desired (crude) product quantified. An example of how to calculate this has been extracted from ICH Q5a (R1) and provided below to illustrate the concept.

Assumptions
Measured or estimated concentration of virus in cell culture harvest = 10^6/ml
Calculated virus clearance factor = $>10^{15}$
Volume of culture harvest needed to make a dose of product = 1 l (10^3ml)

Calculation
[Estimated particles/dose (10^6 virus units/ml) × (10^3 ml/dose)] /clearance factor ($>10^{15}$) = $>10^{-6}$ particles/dose. Therefore, less than one particle per million doses would be expected.

Virus clearance studies are designed based on a model virus selected based on either their appropriateness to the production cell line or a worst-case virus. XMuLV (xenotropic murine leukemia virus-related virus) is typically chosen as a model virus for CHO cell-based manufacturing processes. MMV (mouse minute virus) is typically chosen as the worst-case model virus because (Therapeutic Proteins Global Market Report 2020) it has high chemical resistance and therefore is used to challenge chemical inactivation steps, and (Guidance for Industry and Review Staff 2007) because of its very small size, it can be used to challenge sizing based on removal steps such as the viral filtration step. Once the model viruses are chosen, viral clearance studies are then conducted under reduced scale conditions as it is impractical to execute such studies at the production scale. Virus-spiked feed streams are prepared for selected purification steps and the process step executed at reduced scale. Samples are collected for testing at various process steps (e.g., load, flow through, wash, and elution steps of a bind and elute chromatography step). The amount of virus spiked (vol. in x titer in) is compared to the amount of virus in the product containing pool (vol. out x titer out) yielding a log reduction value ([vol. in x titer in]/[vol. out x titer out]) for the process step evaluated. For a simplistic example, if the amount of virus spiked was 10^6 and the amount of virus in the product containing pool was 10^2, then a 4-log viral clearance value can be claimed for that specific step. The virus quantitation assay is run with the proper positive and negative controls to confirm that the virus titer obtained on the spiked samples or in the process samples is accurate and that the product in or buffer components in the solutions analyzed do not interfere with the viral quantitation assay.

Viral clearance studies are performed on specific steps of the purification process designed to remove or inactivate virus. For antibody products, the protein A chromatography step is a good step to study. First, the mode of interaction is affinity based so that virus particles (which lack affinity to the protein A ligand) will pass through with the flow through and wash. Second, the eluate is in a low pH buffer which acts as a viricidal agent. Genentech performed a meta-analysis on its own viral clearance database of protein A chromatography validation studies from 22 mAbs and 30 processes over the past 15 years; it was observed that clearance of X-MuLV measured by QPCR ranged from 2 to 4 $\log_{10}$ (Zhang et al. 2014). When the protein A affinity step is paired with a low pH hold/inactivation step after the eluate step, developers can expect additional clearance values (Chinniah et al. 2016).

Guideline ICH Q5 A (R1) provides detailed information on viral clearance strategies and methodologies and should be consulted by developers before designing a viral clearance study. It should be noted that health authorities expect such studies be performed in duplicate and that the lower clearance value for each step be used to calculate the overall viral clearance capability of the process.

Conclusions

In the preceding paragraphs, the authors selected and discussed a small subset of CMC development activities that health authorities focus on when reviewing INDs and IMPDs. As a sponsor continues to develop their molecule, the expectations of the health authorities increase as does the amount of information expected in a regulatory filing. Much of that information is usually shared in Sect 3.2.S.2.6 and 3.2.P.2.3 Manufacturing Process Development overview. Information such as the sponsor's experience with the development of each process stage, integrated control strategy, criticality assessment, analytical method history, compilation of all comparability exercises over the life of the molecule, etc., is provided in this section. Given the importance of each topic discussed, it is understandable why these sections contain many hundreds of pages in a marketing application and, for that reason, why the author did not discuss the requirements in any detail. It also bears reinforcing that the expectations of health authorities continue to increase with each filing that is submitted. As such, it is very important that the sponsor have the expertise based on either past filing experience or a detailed understanding of the regulations about the data required in a health authority submission. CMC development and CMC regulatory personnel should map out a content plan for each section of the quality module for each phase of development before performing the development work. Doing so after the work is completed will be too late necessitating the need to generate additional data to fill gaps identified when compiling the regulatory dossier. It takes many years to get a drug into the clinic and then to the market. Adding additional months to the development timeline after a filing is submitted and not accepted will not be viewed favorably. CMC professionals in the biopharmaceutical industry need to spend as much time designing quality into the protocols and reports that are used as source documents for the quality module of a CTA as they do writing the modules themselves. Only by doing so can one expect first time right/approval on their submission.

Cross-References

- ▶ Biomolecules Versus Smaller Chemicals in Toxicology: ICH, EU, and US Recommendations
- ▶ Microbiome Product Toxicology: Regulatory View on Translational Challenges
- ▶ Monoclonal Antibodies: Discovery and Protein Engineering
- ▶ Risk Minimization in Drug Development: Regulatory Aspects
- ▶ Toxicity Testing In Vitro: Regulatory Aspects

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Medical Aspects of Traffic Exhaust Regulations

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Andrea Kaifie and Thomas Kraus

Contents

Abstract

Traffic exhaust emissions are a major driver of premature diseases and deaths. A reduction of traffic exhaust emissions can be supported by a continuous climate-friendly transportation policy. The *European Green Deal* includes such policy measures, for example, the investment in green technologies and cleaner forms of public and private transport. In this chapter, we focus on the adverse health effects of air pollutants caused by traffic exhaust emissions. In particular, low- to middle-income countries but also industrialized regions are affected by traffic-related air pollution. The World Health Organization (WHO) estimated about 4.2 million premature deaths worldwide caused by air pollution. In particular, nitrogen dioxide and particulate matter (PM_{10} and $PM_{2.5}$) are major components of traffic exhaust and can lead to severe health conditions. While nitrogen dioxide has mainly local (respiratory) effects, particulate matter leads to an increase of total and cause-specific mortality. However, European limit values and WHO

A. Kaifie · T. Kraus (✉)
Institute of Occupational, Social, and Environmental Medicine, Medical Faculty, RWTH Aachen University, Aachen, Germany
e-mail: akaifie@ukaachen.de; tkraus@ukaachen.de

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guideline values show a huge disparity with limit values for $PM_{2.5}$. The European limit values are twice as high as the WHO recommendation. Various stakeholders involved in limit value definitions might be a reason for that disparity. Different technical and non-technical measures are introduced to achieve the European climate goal of 2050 – a climate-neutral European Union. Technical measures include particulate filters for light and heavy vehicles and the support of electromobility. Non-technical measures include smart traffic systems, low-emission zones, free public transportation services, and supporting the use of car-sharing services and bicycles.

Keywords

Particulate matter · Nitrogen dioxide · Limit values · Emission · Health

Introduction

The transport sector is the third biggest polluter/perpetrator of greenhouse gas emissions in Germany with almost 20% of all generated pollutants (Bundesministerium für Umwelt Naturschutz und nukleare Sicherheit 2019). Although the federal government supports climate-friendly transportation policies and a significant increase in electric cars on the streets can be seen, only about 63.000 electric cars were officially registered in 2019, compared to 3.600.000 cars powered by combustion technology (Statista 2020; Kraftfahrtbundesamt 2020). A decreasing effect of traffic exhaust emissions can be clearly observed during the current coronavirus pandemic. The coronavirus pandemic leads to a significant reduction in the use of the transport sector resulting in impressive pictures of clean air and blue skies in the world's biggest "smog cities," such as Los Angeles, Jakarta, or New Delhi. As the world still takes a short pause from the significant pollution caused by various economic sectors, a certain sense of optimism for economic recovery is already gaining traction. The federal government of Germany initiated an economic stimulus package to drive consumption behavior. One part of that package includes the sales discount of electric cars. Though unionists and the automotive industry argue and fight that cars with combustion technologies were not included in the stimulus package, the federal government is setting an important marker for the necessity of turning toward a climate-friendly policy. But the discussion about environmental impact of traffic exhaust emissions caused by vehicles with combustion technology is not enough. Traffic exhaust emissions are a major driver of premature diseases and deaths. Though there is clear evidence of an increased mortality associated with traffic exhaust emissions, the impact of its individual components is still part of ongoing research activities. In this chapter, we want to address the different components of traffic exhaust emissions and explain the specific impact on human health. We will discuss what is evident and what is still under debate. Furthermore, we will explain the differences in traffic exhaust regulations, such as the definitions and differences of the terms guidance and limit values

with regard to specific traffic exhaust components. At the end of this chapter, we want to introduce the European legal mechanisms and measures to reduce these emissions.

Air Pollutants

The emission of air pollutants decreased significantly since the 1990s in Germany. Only for ammonia, mostly emitted by the agriculture sector, constant emission values were detected over the last decades. The reduction of pollutant emissions could be observed in all 28 EU member countries including non EU member countries Iceland, Lichtenstein, Norway, Switzerland, and Turkey. However, EU-wide analyses showed an increase of emissions in certain sectors, such as in the shipping (in particular nitrogen dioxide) and in the aviation sector.

For traffic exhaust emissions, carbon dioxide emissions remain on a high level, though efficiency on combustion technologies has been widely implemented. In 2018, the transport sector was responsible for the emission of 162 million tons of carbon dioxide equivalents, compared to 163 million tons of carbon dioxide equivalents in 1990. The self-declared climate goal of the federal government of Germany is to achieve a reduction of carbon dioxide emissions about 40% – by 2030. A reduction of carbon dioxide by implementing sustainable and climate-friendly policy strategies is one of the main recommendations of the Leopoldina National Academy of Science. In particular traffic exhaust leads to health implications beyond the burden with hazardous pollutants (Leopoldina Akademie der Wissenschaften 2019). Besides carbon dioxide, traffic exhaust emissions contain several other components, with particulate matter and nitrogen dioxides as its most prominent representatives.

Particulate matter (PM_{10}, $PM_{2.5}$, ultrafine particles (UFP)) describes the mixture of solid particles and liquid droplets in the air. Particulate matters are emitted ubiquitously. The natural sources of particulate matter are volcanic eruptions, for example. Industrial sources for PM are the transport sector and the agriculture sector among others. PM can be divided into three different groups, depending on the particle size. PM_{10} has a diameter less than 10 μm, $PM_{2.5}$ has a diameter less than 2.5 μm, and ultrafine particles (UFP) have a diameter less than 0.1 μm. The particle diameter has a significant influence on the distribution in the human body. For example, PM_{10} remains mainly in the upper respiratory tract, while $PM_{2.5}$ can penetrate the lower respiratory tract (alveoli). UFP are able to pass the air-blood barrier and can induce systemic health effects. PM can be further divided into primary and secondary particulate matter. Primary particulate matter is emitted directly into the atmosphere. Secondary particulate matter is formed by photochemical reactions of different molecules in the atmosphere. The health impacts of PM depend on the particle size and surface which is the crucial factor for the penetration depth in the body and the composition of the PM. Secondary PM can take up hazardous components, such as heavy metals or organic pollutants with carcinogenic

potential. If these components have a particle size less than 0.1 μm, they can pass the air-blood barrier and distribute in different organ systems.

Black carbon (BC) is a component of particulate matter and is formed by the incomplete combustion of fossil fuels. Black carbon is co-emitted with other particles and gases. Main sources for black carbon emissions are open burnings, traffic, and heavy industry. Black carbon has a significant environmental impact by driving global warming. Regarding health impacts, BC can be assigned to the PM group with its specific health effects, depending on the particle size.

Nitrogen monoxide or dioxide (NO/NO_2) belongs to the group of gases called nitrogen oxides (NO_x) and is mostly emitted by burning fossil fuels. Regarding the impact on human health and environment, NO_2 is of great concern. Vehicles using diesel are the main sources for NO_2 in road traffic. Aside from the transportation sector, power plants are a main issuer of NO_2 emissions. Discussions about driving restrictions usually focus on NO_2 emissions, although health impacts are mostly restricted to the respiratory system. The environmental impact of NO_2 and other NO_x is the formation of acid rain with its dramatic implications on sensitive ecosystems, such as forests.

Ozone (O_3) is an odorless gas and a so-called "secondary" pollutant. It is formed from gases, such as nitrogen oxides or volatile organic compounds in the presence of solar light. Since nitrogen dioxides are the basis for the formation of ozone, nitrogen oxide-emitting sources are the cause for high ground-level ozone concentrations. High ozone concentrations are dependent on the season, since high levels of solar radiation and high temperatures during summer lead to its formation. Ozone can lead to an irritation of the airways. In particular in persons with underlying respiratory conditions, high levels of ozone can lead to severe clinical symptoms.

Sulfur dioxide (SO_2) is a toxic gas with a typical acrid smell. Although sulfur dioxide can originate from natural sources, about 99% of the sulfur dioxide in the air comes from industrial processes, such as industrial heating installations and power plants. Sulfur dioxide is mostly responsible for acid rain with its environmental implications for water, soil, and forests. The human health impact of SO_2 is, similar to ozone, the irritation of the respiratory system.

Health Effects

Ambient air pollution is a health risk, and traffic exhaust emissions are a major contributor to ambient air pollution. The World Health Organization (WHO) states that reducing air pollution can lead to a decrease in the prevalence of cardiovascular diseases, such as stroke or coronary heart disease, acute and chronic respiratory diseases, as well as lung cancer (World Health Organization 2018). In 2016, more than 90% of the world's population was living in places, where the recommended WHO guideline values for air pollutants were not met. In particular, low- to middle-income countries are affected by air pollution causing about 4.2 million deaths worldwide, the WHO estimated. While air pollution caused by traffic exhaust emissions contains several different components, we want to focus on the two

substances that play a major role in assessing and estimating health consequences – nitrogen dioxide (NO_2) and particulate matter (PM_{10} and $PM_{2.5}$). For the evaluation of the health effects, toxicological as well as epidemiological data have to be taken into account.

Nitrogen Dioxide (NO_2)

For nitrogen dioxide, several animal and human exposure studies examined the impact of this particular pollutant on health. In *animal studies*, rats have been exposed to NO_2 via inhalation which led to the definition of a workplace limit value of 950 μg/m^3. Several other inhalation studies in animals showed toxic changes at concentrations of more than 3000 μg/m^3. *In vitro studies* showed cytotoxic effects on bronchial epithelial cells at concentrations more than 7520 μg/m^3. NO_2 can damage proteins and lipids in vitro, newer studies showed (Beratungskommission der Gesellschaft für Toxikologie 2019).

In *human exposure studies*, study participants were short-time exposed to NO_2 under controlled conditions. NO_2 levels exceeding 1900 μg/m^3 led to an impairment of the respiratory function. Persons with respiratory preconditions showed already an impairment of the respiratory function at NO_2 levels of 560 μg/m^3 (Beratungskommission der Gesellschaft für Toxikologie 2019).

Regarding short-term and long-term exposure to NO_2, several *epidemiological studies* examined and evaluated potential health effects (Beelen et al. 2014; Brown A.C. Health Effects of Particulates and Black Carbon 2013; Goodman et al. 2009; Agency EE 2019; US Environmental Protection Agency 2016; Folinsbee 1992; Heinrich et al. 2013; Hoek et al. 2013; Gehring et al. 2015). Short-time exposure to NO_2 can lead to respiratory effects, such as the worsening of symptoms, in particular in persons with respiratory preconditions. The worsening of respiratory symptoms can lead to higher hospital admission rates. Regarding long-term effects of NO_2, the EEA (European Environment Agency) and the WHO evaluated the effects differently in comparison to the US-EPA (Unites States Environmental Protection Agency). For the EEA, long-term exposure to NO_2 may increase the mortality (Agency EE 2019). However, adjustment on other pollutants has not been carried out. The US-EPA only describes a *suggestive evidence* between NO_2 exposure and total mortality. Same applies to other effects outside the respiratory system, such as cardiovascular effects, diabetes, birth outcomes, and cancer (US Environmental Protection Agency 2016). The US-EPA states that nitrogen dioxide is unlikely to enter the bloodstream and cause systemic effects outside the respiratory system. Furthermore, the potential influence of other traffic-related pollutants has not been taken into account. In the analyses of traffic exhaust emissions, a clear correlation between NO_2 and black carbon (BC) as well as other UFP could be observed (Wichmann 2018). In epidemiological studies, a differentiation between those components has not been carried out (US Environmental Protection Agency 2016). Therefore, it is difficult to differentiate between the NO_2 effect and the effect of the other components. The elevated mortality in some studies

Table 1 Short-term and long-term effects of nitrogen dioxide and particulate matter

Pollutant	Short-term exposure	Long-term exposure
Nitrogen dioxide (NO_2)	• Respiratory effects, such as the worsening of symptoms in particular for persons with respiratory preconditions • Higher hospital admission rates • (Increase of mortality)[a]	• Respiratory effects, such as development of asthma, exacerbation of respiratory symptoms • (Increase of mortality)[a]
Particulate matter ($PM_{2.5}$)	• Effects on the respiratory and cardiovascular system • Increase of mortality	• Increase of total, cardiovascular, and respiratory mortality

[a]EEA, WHO only

could therefore be assigned to the exposure to UFP or other compounds and not NO_2. However, NO_2 is a surrogate marker for traffic exhaust emissions, and a reduction in the NO_2 air concentrations should be definitely a policy goal. Short-term and long-term effects of NO_2 are summarized in Table 1.

Particulate Matter

While EEA, WHO, and US-EPA came to varying conclusions regarding NO_2 long-term exposure, the evaluation of the impact of particulate matter is more unified. Short-term exposure to particulate matter affects the respiratory and cardiovascular system and leads to an increase of mortality, as well (Agency EE 2019; Wichmann 2018; US Environmental Protection Agency 2019). The short-term effects are supported by positive associations for the cause-specific mortality. Cause-specific mortality includes cardiovascular and respiratory mortality. Fatal cardiovascular events that increase the mortality are ischemic events and heart failure. Fatal respiratory events include the exacerbation of COPD or asthma. The long-term effects include an increase of cardiovascular, respiratory, and total mortality (Table 1) (Leopoldina Akademie der Wissenschaften 2019; Heinrich et al. 2013; Hoek et al. 2013; Wichmann 2018; US Environmental Protection Agency 2019; Kaifie et al. 2019; European Environmental Agency 2019; Chen et al. 2008). The increase of mortality is classified as *causal* by the US-EPA (US Environmental Protection Agency 2019). The associations between long-term exposure to $PM_{2.5}$ and mortality remained significant, even after controlling for ecological and individual covariates, such as socioeconomic status and smoking habits. Similar to the short-term exposure, the total mortality is supported by positive associations for cardiovascular (i.e., coronary heart diseases, stroke), respiratory (i.e., COPD), and lung cancer mortality (US Environmental Protection Agency 2019). A decrease in $PM_{2.5}$ exposure leads to an increase in life expectancy, studies showed (US Environmental Protection Agency 2019).

Further relationships between $PM_{2.5}$ exposure and the development of certain diseases have been evaluated by the US-EPA, as well. Regarding long-term exposure

to $PM_{2.5}$ and nervous system effects, the US-EPA described a *likely to be causal* relationship. Toxicological studies in animals showed nervous system effects, such as neuroinflammation, neurodegeneration, cognitive effects, and effects on neurodevelopment (US Environmental Protection Agency 2019). The toxicological data were supported by epidemiological studies that detected changes in brain morphology, cognitive decrements, and dementia after long-term exposure to $PM_{2.5}$. In addition, there is limited epidemiological evidence that long-term exposure to $PM_{2.5}$ is associated with autism spectrum disorders (US Environmental Protection Agency 2019).

The relationship between long-term exposure to $PM_{2.5}$ and cancer is classified as *suggestive of a causal relationship*. $PM_{2.5}$ is leading to genotoxic and epigenetic effects and has carcinogenic potential. Genotoxic effects include DNA damage, epigenetic alterations can lead to hypo- and hypermethylation of DNA, and cytogenetic effects and alterations in gene expressions can promote cancer pathogenesis. Further $PM_{2.5}$ effects are the induction of oxidative stress and electrophilicity. Regarding lung cancer, US-EPA classified this relationship as *likely to be causal*. In particular, the positive associations between $PM_{2.5}$ exposure and lung cancer in never smokers underlined that assessment (US Environmental Protection Agency 2019).

The German Respiratory Society supports further pathogenic effects that can be related to general exposure to air pollutants. Those effects include the development of diabetes mellitus and effects on pregnancy and fetus. In particular vulnerable groups, such as pregnant women, children, or elderly, are under particular risk by air pollution (Schulz et al. 2018).

The development of diabetes mellitus may be caused by the continuous activation of systemic inflammation that leads to inflammatory effects in the liver, muscle, central nervous system, and fat tissue. Eventually, these inflammatory processes can lead to disorders in the carbohydrate metabolism (Schulz et al. 2018).

For pregnancy and the fetal development, potential effects are disturbances in placenta growth and function, as well as alterations in the maternal circulation. Potential effects on the mother may be hypertension, pre-eclampsia, and disorders of placental growth and function. A reduced fetal weight, premature births, and stillbirths are potential fetal effects. For infants, a reduced respiratory function as well as allergic diseases and disorders is described (Schulz et al. 2018). All potential effects of air pollution are summarized in Fig. 1.

Guidance Values Versus Limit Values

Though the health impact of air pollutants is highly relevant, political implication does not necessarily reflect these findings. The differences in guidance values and limit values underline this statement. The WHO guidance values mark the limit for certain pollutants, in which exceedance can lead to health effects. The guidance values are generated under consideration of toxicological as well as epidemiological studies. Health experts reviewed all studies and determined the concentration which

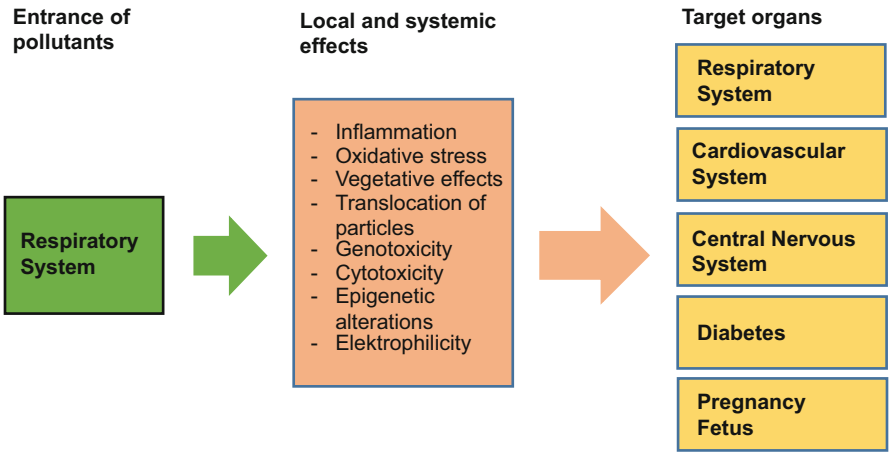

Fig. 1 Summary of potential air pollution effects. (Based on information of Schulz et al. 2018)

Table 2 Limit and guidance values of $PM_{2.5}$, PM_{10}, and NO_2. (Based on informations of European Commission 2019)

Pollutant	EU limit values	WHO guidance values	Averaging period
$PM_{2.5}$	–	25 µg/m^3	24 h
	25 µg/m^3	10 µg/m^3	1 year
PM_{10}	50 µg/m^3	50 µg/m^3	24 h
	40 µg/m^3	20 µg/m^3	1 year
NO_2	200 µg/m^3	200 mg/m^3	1 h
	40 µg/m^3	40 µg/m^3	1 year

should not be exceeded in order to avoid adverse health effects. The country-specific limit values also include a political dimension and can be defined as a consent between different stakeholders. The main disparity between these values is the consequences followed by an exceedance of these values. While an exceedance of limit values has legal consequences, guidance values are only recommendations. An exceedance of guidance values has no consequences for the particular country. The following table (Table 2) summarizes the guidance values of the WHO as well as the limit values of the European Union.

While limit and guidance values for nitrogen dioxide are similar, there is a huge disparity for particulate matter. The WHO recommends not to exceed an annual concentration of 20 µg/m^3 for PM_{10}, while the EU limit value is twice as high with 40 µg/m^3. For $PM_{2.5}$, the EU limit value is even 2.5 times higher than the recommended guidance value of the World Health Organization. In particular, particulate matter with a small diameter, such as $PM_{2.5}$, can penetrate the air-blood barrier and enter the circulatory system. Therefore, this huge disparity is difficult to understand, under consideration of the systemic health effects of particulate matter

with an increase of total and cause-specific mortality (cardiovascular and respiratory mortality). The German Respiratory Society states that with the current limit values, health effects on the population can be expected. This is of particular interest for the previously described vulnerable groups, such as pregnant women, children, and elderly.

For nitrogen dioxide, the accordance of limit and guidance values is a progress. Though nitrogen dioxide is most likely not increasing mortality rates after long-term exposure, the short-term adverse health effects are significant, such as respiratory exacerbations and an increase in hospital admissions. In the USA, the limit value for nitrogen dioxide is set at 100 $\mu g/m^3$ for long-term exposure. This reflects the evidence of the US-EPA that observes local effects for NO_2, such as an exacerbation of symptoms in patients with respiratory preconditions or the development of asthma. However, NO_2 is also a surrogate marker for other traffic exhaust emissions, such as ultrafine particles or black carbon. Epidemiological studies were not able to differentiate between the particular health impacts of the single components so far. Therefore, a low limit value for nitrogen dioxide should be sought. In contrast to nitrogen dioxide, the limit value for particulate matter ($PM_{2.5}$) in the USA is set to 12 $\mu g/m^3$, less than half compared to the EU limit value and in closer accordance to the WHO guideline values.

The guidance values of the WHO were determined by scientific evidence under consideration of all available experimental and epidemiological data. The question is why are the EU limit values for particulate matter so much higher than the guidance values of the WHO though the adverse health effects of particulate matter are evident? The reasons for that can be found in a communication letter from the European Commission (EC). The EC states: "For particulate matter, compliance with EU limit values was due as of 1 January 2005. Concentrations of particulate matter continue to exceed these values in large parts of Europe – with exceedances reported in 19 out of 28 Member States. Based on the latest available data, 19% of the urban population in the EU have been exposed to particulate matter levels above the EU daily limit value and about half was exposed to concentrations exceeding the stricter recommendations by the World Health Organization" (European Commission 2018). That statement clearly indicates that several EU member states were not able to comply with the specific EU limit values for particulate matter. An exceedance of limit values has legal consequences. The European Court of Justice is already prosecuting several EU members due to the exceedance of air pollution limit values. A tightening of limit values, as suggested by the WHO, would lead to further violations of the EU Directive for clean air by the EU member states. The decision, to keep the limit values at that level, can be clearly stated as political intention in order to avoid an endless number of legal proceedings against EU member states. However, it is the duty of the EU administration to keep their citizens healthy. Therefore, it is urgently required to stepwise approximate the EU limit to the WHO guidance value of 10 $\mu g/m^3$ for $PM_{2.5}$.

The EU has several mechanisms to promote a climate-friendly policy in order to reduce air pollution via traffic exhaust emissions. The legal mechanisms of the EU are further described in the next paragraph.

Legal Mechanisms

Ursula von der Leyen, president of the European Commission, stated in July 2019 that she wanted Europe to be the first climate-neutral continent in the world to make the EU completely carbon neutral by 2050. In December, she presented the *European Green Deal* to the EU leaders in order to initiate a green transition. To achieve a climate-neutral EU by 2050, a European climate law was proposed to turn the commitment into legal obligation. The required actions to achieve this goal include:

- Investing in green technologies
- Cleaner forms of public and private transport
- Support energy efficiency of buildings
- Decarbonizing the energy sector

To achieve these goals, international cooperations on a global basis are necessary. However, several other legal mechanisms have been implemented by the EU, so far. In May 2008, the Directive 2008/50/EC on ambient air quality and cleaner air for Europe has been introduced (European Parliament 2008). The main goal of this directive was to reduce pollution to minimize adverse health effects and to pay attention to vulnerable populations and the environment as a whole. Mechanisms to reduce the air pollution included the combat of emissions on local, national, and community level. The critical pollutants were defined and included sulfur dioxide, nitrogen dioxide, and oxides of nitrogen, particulate matter (PM_{10} and $PM_{2.5}$), lead, benzene, and carbon monoxide. All EU member states were obligated to assess ambient air quality with regard to these pollutants.

In 2013, a Clean Air Program for Europe has been set up that efforts rested on three pillars. The first pillar included ambient air quality standards that were to be attained by all member states. The second pillar focused on national emission reduction targets, in particular for substances, such as sulfur oxides, nitrogen oxides, ammonia, volatile organic compounds, and particulate matter. The third pillar comprised emission standards for key sources of pollution, such as vehicle emissions and industrial processes. For vehicle emissions, the Regulation (EC) No 443/209 setting CO_2 emission standards for new passenger cars and Regulation (EC) No 510/2011 setting CO_2 emission standards for new light commercial vehicles, and Regulations on CO_2 emission standards for new cars and light duty vehicles have been introduced.

Reflecting the important contribution of traffic-related air pollution, local and regional clean air plans have been implemented. These included setting of limit values for traffic-related pollutants and limits on total emission as well as setting emission standards, such as Euro emission standards 1–6 or by setting requirements for fuel quality. After the vehicle emission scandal in 2015, driving emission rules were put in place to ensure air pollutant emission standards were properly implemented, in particular for nitrogen oxides and particulate matter.

Other mechanisms in order to reduce traffic exhaust emissions include technical measures, behavior change and demand management, and infrastructure investment (European Commission 2018). In 2017, the European Commission introduced two Mobility Packages. The first Mobility Package included rules on road pricing and extending the European focus on buses, vans, and passenger cars. The second Mobility Package included measures for a cleaner public transport or the shifting to low- and zero-emission vehicles through new carbon dioxide standards for cars and vans. Another important aspect is infrastructure guidelines for alternative fuels that included charging opportunities for electric cars. A third Mobility Package focused on carbon dioxide emission standards for heavy vehicles as well as the establishment of a Europe-based battery manufacturing industry. Those batteries are intended to be included into low- and zero-emission vehicles. The EC is furthermore supporting local administrations in addressing aspects of vehicle access restrictions to foster consistency and information on regulation. Sustainable Urban Mobility Plans were already included in the Directive 2008/50/EC.

Technical Measures

The vehicle emission scandal uncovered the manipulations of the automotive industry using illegal technical measures to maintain exhaust emissions below the limit values, in particular nitrogen dioxide and carbon dioxide. A vehicle using an illegal switch-off design is frequently emitting a higher amount of pollutants; therefore, a software solution was the initial measure to reduce NO_2 and CO_2 emissions.

Besides the use of software solutions, further technical measures are necessary in order to reduce traffic exhaust emissions. Here, the installation of catalytic converters and diesel particulate filters represents two possible mechanisms. Particulate filters with closed systems can reduce the emission of particulate matter around 90%. To support this technical measure, a law concerning retrofitting diesel cars has been passed in 2007. Owners of diesel cars were financially supported if they install diesel particulate filters in their cars. In addition, financial incentives have been implemented to support the use of low-emission heavy vehicles. Heavy vehicles with low traffic exhaust emissions pay less road pricing rates. For Germany, the government financially supports the purchase of low-emission trucks.

A cleaner public transport using technical measures comprises a further important measure to reduce air pollution by the transport sector. The installation of particulate filters in busses and the transition to electromobility have been implemented in several locations. In San Francisco, the San Francisco Municipal Railway maintains the worldwide second largest trolleybus system. Governmental financial support in purchasing and maintaining electric private or public transport vehicles is a useful measure to reduce traffic exhaust emissions in the urban setting.

Non-technical Measure

Several non-technical measures have been implemented to reduce traffic exhaust, so far. The main burden from air pollutant emissions can be found in cities; increasing urbanization will reinforce that problem. Therefore, sustainable strategies to improve the air quality in cities are urgently needed.

In Germany, environmental or low-emission zones have been created for big cities with air pollution problems. Altogether, there are 58 environmental zones, in particular in metropolitan areas, such as North Rhine-Westphalia and Baden-Württemberg. Only cars with a green sticker that indicates a low-emission vehicle can travel freely in these environmental zones. A green sticker covers a category of vehicles with the lowest particulate and harmful emissions level. The other three of altogether four categories are indicated by the colors yellow and red and no sticker. Vehicles without a catalytic converter and old diesel cars are not allowed to enter the low-emission zones. Low-emission zones have not only been established in Germany but in whole Europe covering more than 300 environmental zones in 13 different countries.

Besides low-emission zones, speed restrictions are a further measure to reduce air pollution by traffic exhaust emissions. Speed restrictions lead to a reduction of particulate matter and nitrogen dioxide emissions. As the Leopoldina National Academy of Science stated, traffic exhaust leads to health implications beyond the burden with hazardous pollutants (Leopoldina Akademie der Wissenschaften 2019). Those health implications include adverse effects through noise as well as (deadly) road accidents, which can all be reduced by speed restrictions.

Smart traffic systems to reduce air pollution may be another non-technical measure. One option is the construction of peripheral roads in order to reduce the high traffic load in certain urban areas. A smart traffic light system might be able to decrease the particulate matter emissions by reducing repeated braking and accelerating which leads to high emissions with air pollutants. Financial incentives to use autonomous driving constitute a further useful measure.

Governmental financial support to use public transport services has been implemented already in several European countries. In Latvia, the public transport services can be used for free for several years. Monheim in North Rhine-Westphalia introduced free public transport services in 2020 since only 10% of the population used busses, so far. In Pfaffenhofen in Bavaria, free public transport services lead to a doubling of passengers using busses. However, more detailed analysis revealed that free public transport services are frequently used by passengers who previously walked or used their bicycle. So far, only a minority switched from car to bus. Free public transport services are therefore only able to a limited extend to get car drivers off the street. Car-sharing services could therefore be a more low-threshold measure to reduce urban air pollution. Car drivers can stay with their habitual use of cars, but sharing services lead to a reduction of total car density on the street. The improvement and expansion of bicycle tracks are a further measure to achieve such a goal. As weather conditions and geographical features may play a significant role in the regular use of bicycles, further studies need to evaluate the success of such a

measure. However, there are several possibilities to reduce in particular the urban traffic load. The political momentum and initiative are essential for a climate-friendly traffic policy, in particular in an automotive country, such as Germany.

Cross-References

- ▶ Exposure Analysis for Indoor Contaminants
- ▶ Limit Values and Guideline Values in Regulatory Toxicology
- ▶ Nanoparticles and Their Regulation
- ▶ National and International Collaboration in Regulatory Toxicology
- ▶ Risk Communication: Challenges for Toxicologists and Other Risk Experts
- ▶ Toxicokinetic Tests

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Tattooing: Toxicology and Risk Assessment 90

Michael Giulbudagian, Sandra Wagener, Peter Laux, and Andreas Luch

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Abstract

Driven either from self-expressing, religious, or cosmetic motivations, tattooing has been a long-lasting practice for centuries and will probably remain being for the years to come. Permanent body decorations are not anymore allied with sailors, prisoners, or soldiers only but rather penetrated into the mainstream of modern society. Given the rising popularity, research interest has been directed to assess possible health effects caused by the injection of substances into the human dermis. The regulatory side has in parallel put efforts for identifying the most appropriate measures for protecting public health. Here, we provide a recent literature update on tattoo toxicology. Further, the milestones leading to the first European-wide regulation of substances in tattoo inks and permanent makeup are presented.

Keywords

Tattoo · Permanent makeup · Risk assessment · Restriction

M. Giulbudagian (✉) · S. Wagener · P. Laux · A. Luch
Department of Chemicals and Product Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany
e-mail: Michael.Giulbudagian@bfr.bund.de; Sandra.Wagener@bfr.bund.de; Peter.Laux@bfr.bund.de; Andreas.Luch@bfr.bund.de

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Introduction

Tattoos and permanent makeup (PMU) have become very popular during the last decades. The tattooed population comprises people of different ages and cultures estimated up to 12% in the EU and 33% in the USA, with slightly higher percentages in the younger ages of 18–29 years (Bäumler 2016; Breuner et al. 2017; JRC 2016; Renzoni et al. 2018). Tattoo inks contain a large number of ingredients, including pigments, binders, solvents, and additives (Giulbudagian et al. 2020). A major concern in the context of pigments used for tattooing is the fact that they are mostly manufactured for other than tattooing applications, e.g., the automotive, construction, or cosmetics industries (Laux et al. 2016). Even if their usage in numerous products may be considered safe, their intradermal application has never been tested (Foerster et al. 2020). Hazardous substances are still detected recurrently in tattoo inks (Foerster et al. 2020; Laux et al. 2016; Schreiver et al. 2015). Their hazard classification mostly refers to the topical, oral, or respiratory uptake, but not to intradermal exposure, which however may cause a different kind of toxicity. This is also an obstacle in terms of tattoo ink risk assessment. Strategies for the identified substances rely on the evaluation of chemical hazards identified by oral, topical, or inhalation administration. These data cannot easily be extrapolated to subcutaneous use.

Despite this alarming situation, it has to be noted that most people remain without severe complications (JRC 2016). This especially applies to systemic effects. While local reactions can rather easily be assigned to the site of the tattoo, postponed or chronic effects appearing on remote organs are challenging for attribution. As substances in the inks are subject to metabolism upon their injection, the identification of the exact molecular fragments responsible for the observed reaction is inevitable for assessing their risks. These facts together with the widespread use have encouraged scientific as well as regulatory efforts to identify possible health effects and to develop a risk assessment strategy of the tattoo inks.

This chapter describes the state of knowledge on adverse effects following tattooing and introduces approaches on the risk assessment of tattoo inks.

Health Risks of Tattoos: Clinical Evidences

Health risks as a consequence of tattooing are diverse and can be attributed to different causes, modes of actions, as well as different sites of the human body. Triggers of possible adverse effects may be the tattooing process as such, the ingredients of the tattoo ink, as well as decomposition products following metabolism of the pristine substances within the body. Adverse effects can occur immediately after tattooing but also weeks or years later. Associated therewith, effects may appear on the tattooed site, the surrounding region, or other organs after systemic distribution of the respective compounds (Fig. 1). Nonetheless, evidences for the latest are mostly limited to observations in animals.

Immediately after the tattooing process, acute aseptic inflammation may occur comprising swelling and erythema, together with transient bleeding and lymphadenopathy

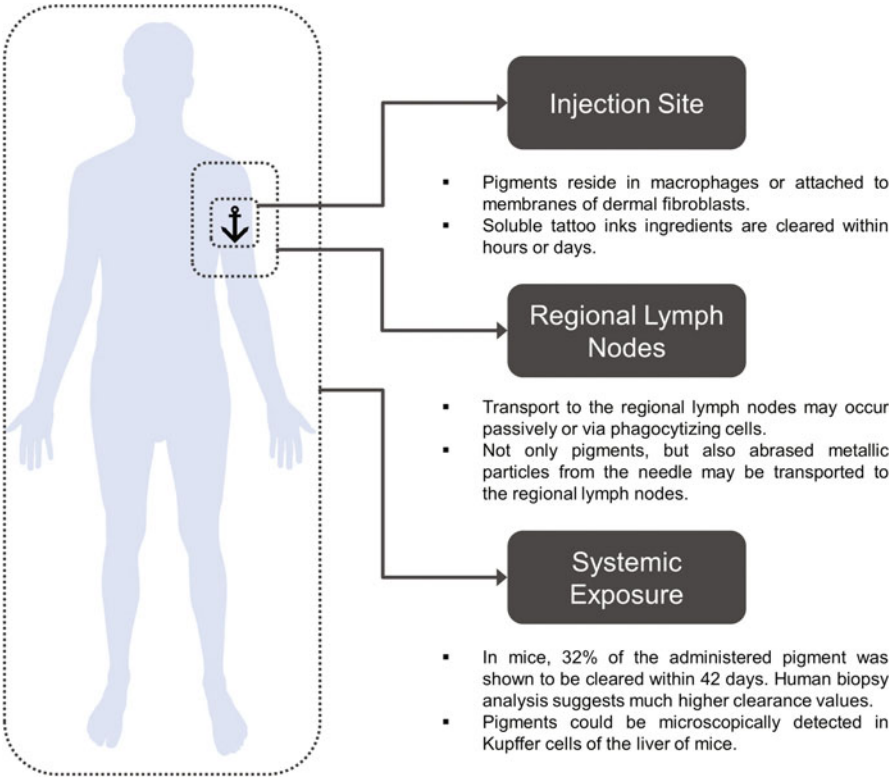

Fig. 1 Depiction of a tattoo and its local, regional, and systemic features

(Kluger 2010). The healing process lasts about 1–4 weeks. The crusted lesion may provoke pain, itching, blistering, and burning sensation. The sensation of severity of these symptoms is individual. In a German internet survey conducted in year 2010, people described the severity between negligible and very intense (Klugl et al. 2010). Apart from these non-infectious indications, infectious risks provoked by bacteria and viruses have been described. Bacterial infections concern about 1–5% of the tattooed people. To be mentioned here is the occurrence of *Staphylococcus* spp. (e.g., *Staphylococcus aureus*), *Streptococcus* spp. (e.g., *Streptococcus pyogenes*), mycobacteria (nontuberculous and tuberculous), and pseudomonads (JRC 2016; Laux et al. 2016; Rodriguez-Blanco et al. 2011). Causes for infections are diverse, potentially arising from the tattoo artist, non-sterile instrumentation or inks, as well as infections during the wound healing process (JRC 2016). According to Baumgartner and Gautsch (2011) and Høgsberg et al. (2012), up to 20% of sampled inks were found to be contaminated, even when they have been labelled as sterile. Of particular concern, they mentioned the environmental pathogen *Mycobacterium chelonae* (Hogsberg et al. 2013; Kennedy et al. 2012; Rodriguez-Blanco et al. 2011). Clearly more seldom are infections due to fungi or viruses after

professional tattooing. Only in exceptional cases, severe effects such as pyogenic infection with abscess formation, cellulitis, or gangrene may occur and can develop into systemic and life-threatening infection, like sepsis or endocarditis (Laux et al. 2016).

Components

Tattoo inks are being composed of pigments, binders, solvents, and additives. It is estimated that they comprise up to 100 different chemicals (Foerster et al. 2020). The diversity of ingredients together with the occurrence of possible impurities like primary aromatic amines (PAAs), nitrosamines, or various polycyclic aromatic hydrocarbons (PAHs) impedes the search for effects and their causes. The formulations used for tattooing might further contain phenols, formaldehyde, or phthalates. While the development of acute inflammatory reactions, as described before, is frequently a consequence of the tattooing process itself, adverse reactions arising from different ingredients cannot be excluded. For instance, skin reactions were slightly more frequent when colored rather than when black ink was tattooed. This may be attributed to the decomposition of organic pigments (Klugl et al. 2010). Serup et al. (2015) further reported soft tissue lymphatic edema simultaneously occurring with a pigmentation of the surrounding skin and sometimes of regional lymph nodes. He further considered papular or nodular thickening and elevation in specific tattoo areas as a result of pigment overload.

The vast majority of complications that can be traced back to the tattoo ink are related to hypersensitivity, primarily linked to colored inks, rather than to black pigments. As was carried out in a study of patients who had been tested positive for allergic tattoo reactions (Serup and Carlsen 2014), the primary allergen identified was nickel ($n = 16$ out of 90 probands). This element represents a contaminant not intentionally added to tattoo inks. Allergic reactions to nickel were followed in frequency by four positive results for pigments and two for dyes normally used in textiles. The red color was reported often with relation to allergic skin reactions. Patients with positive reactions did not respond to patch tests of the respective pigment; the source of allergic reaction was seen in the formation inside the skin, probably through metabolism, haptenization, or both. As was analyzed in a further study of Serup et al. on 104 dermatome biopsies, P.R. 22, P.R. 170, and P.R. 210 were determined as the predominating pigments behind chronic allergic reactions in red tattoos (Serup et al. 2020). The authors concluded that the reactions can be traced back to a pigment degradation product. They further found elevated levels of different metals like Fe, Cr, or Ni; however, their contribution to allergic reactions in red tattoos could not be proven.

A new finding requiring attention was recently revealed by Schreiver et al. (2019). They found that the needle may release nano- and micrometer-sized nickel (Ni) and chromium (Cr) particles during the tattooing procedure, which is promoted if the ink contains abrasive materials like titanium dioxide. These particles were

found in the skin as well as in the local lymph nodes. These findings are important concerning the assessment of Ni-driven allergies arising from tattoos.

The cleavage of pigments in general is an important issue of concern. Generally, aromatic chromophores may underlie photodecomposition. Some of these decomposition products are suspected or known human carcinogens. Examples comprise pigment yellow 74, decomposing to various primary amines or orange 13, decomposing to substances such as 3,3′-dichloro-4-aminobiphenyl or 3,3′-dichlorobenzidine (Cui et al. 2004). Together with the aforementioned substances like PAAs or PAHs, including benzo[*a*]pyrene, which may occur in colorants, a number of substances of known carcinogenic potential come in contact with human blood and lymph tissue upon tattooing. In this context, it is comprehensible to investigate if tattooing increases the risk of developing cancer. Even though this research is in the beginning and epidemiological studies are rare, a closer examination of the biodistribution of substances may give first insights.

As tattoo inks come into direct contact with blood and lymph fluids, they must be considered as 100% systemically bioavailable (Giulbudagian et al. 2020). Especially for the soluble ingredients such as preservatives, conditioners, or solubilizing additives, participation in metabolic processes is supposed. The solubilizing additives may present up to 70% of the ink and become systemically bioavailable right after the tattooing process (Sapota et al. 2003). It cannot be excluded that this short-term exposure, temporarily reaching very high amounts, could increase the risk to develop cancer on long term (Foerster et al. 2020).

As for pigments, some scientists believe that the low solubility renders them to be biologically inactive (Engel et al. 2006). This argument is underlined by the persistence of the tattoo coloring at the site of administration. However, it cannot be excluded that even a slow metabolism is relevant. Further, insoluble pigments could be detected in regional lymph nodes (Gopee et al. 2005) of SKH-1 mice and human biopsies. Using the same mouse model, the transport and decomposition of P.R. 22 was investigated by Engel et al. (2010). They showed that 32% of the pigment was cleared from the site of injection. After exposure to simulated solar radiation the cleared amount increased to >60%. While some of the fading may be allocated to wound healing (Foerster et al. 2020), the results indicate a degradation of the pigment followed by clearance of the metabolites. An even higher clearance rate of about 87–99% directly after tattooing was estimated by Lehner et al. (2011) from human skin biopsies. Even if this value might be overestimated, there is clear evidence that the lymphatic system plays a major role in the transport/migration of pigments. In support of this theory, Lehner et al. (2014) detected PAHs, most probably associated to carbon black ink, simultaneously in skin samples (0.6 μg/cm^2) and in lymph nodes (11.8 μg/g).

In a study of Sepehri et al. (2017) on the systemic distribution of tattoo pigments, several organs of mice tattooed with black and red inks were analyzed. While in most of the organs pigments were absent, some could be identified in lymph nodes and in Kupffer cells of the liver, suggesting pigment distribution via the bloodstream. In this study, pigments could not be detected in any other organs. However, according to Gopee et al. (2007) and Tang et al. (2009), evidence of the deposition in other

organs such as the liver, spleen, kidney, and hepatic lymph nodes following intradermal and subcutaneous injection of particles is given.

So far, the causal link between tattooing and tumor formation could neither be established nor excluded, which is most likely explained by their long latency and their multifactorial character (JRC 2016). According to Kluger (2010) who reviewed studies of the last 40 years, the small number of 50 tumors on tattoos appears negligible compared to the millions of tattooed individuals and the millions of skin cancers per year (Serup et al. 2015). The number making the association between the two events is rather coincidental. Epidemiological studies may deliver valuable data for understanding effects on the systemic level. Numerous case studies have reported on tumors co-localized with the tattoos (Kluger et al. 2017; Kluger and Koljonen 2012). Although tattoo products may contain carcinogenic substances, no clear evidence has been provided for a causal relationship between cancer and tattoos. A recent study examined the prevalence of basal cell carcinoma in a population with cosmetic tattoos, also known as permanent makeup (PMU) (Barton et al. 2020). The total cohort size was 1745 persons in an age range of 25–50 years. In particular, it was investigated whether early basal cell carcinoma was related to the location and color of the tattoo. The adjusted odd ratio of basal cell carcinoma co-located with a tattoo was 1.8 compared to other parts of the body. The authors concluded that the development of an early basal cell carcinoma is particularly common among yellow and green tattoos. However, due to the lack of baseline characteristics of the study, the causal effect of tattoos on early basal cell carcinoma remains questionable (Kluger and Dub 2020). In particular, the anatomical site of the carcinoma was not defined as being developed on the tattoo itself. Moreover, the calculation was based on people who wore a tattoo and were diagnosed with basal cell carcinoma, leaving out the non-tattooed population. Since the area of the skin covered or the tattooing agents used were not considered in the study, no dose-response affiliation could be derived.

Risk Assessment

Common toxicological and regulatory tools, which rely on the evaluation of chemical hazard administered by oral or cutaneous pathways, supply only limited information for the assessments of risks associated with tattoos. In addition to tattooing, the injection of substances into the human dermis is also being used for the application of PMU by procedures termed as micro-blading or micro-shading. Common pigments used in PMU are different compositions of iron oxides in shadows of yellow, red, brown, or black. Although differing somewhat from tattooing in terms of the pigments used or the amount and depth of application, these have to be treated equally for the purpose of risk assessment and regulation. Moreover, the common to all pigments used for tattooing or PMU applications is their very low solubility in water as well as in many organic solvents. Hence, the distinction in terminology of pigments (particles) and their water-soluble analogs referred as dyes is crucial. Nevertheless, dyes may be converted into non-soluble pigments by precipitating them with various metals, referred as lakes.

Table 1 Selected requirements for the safety assessment of tattoo and PMU inks as assessed by the German Federal Institute for Risk Assessment (BfR) and the European Directorate for the Quality of Medicines & Health Care (EDQM) (BfR 2012; EDQM 2017)

Physicochemical characterization	Toxicological endpoint assessment
1. Chemical identity	1. Skin irritation
2. Physical form	2. Irritation to mucous membranes
3. Molecular weight	3. Phototoxicity Photo-sensitization
4. Characterization and purity of the substance	4. Sensitization
5. Characterization and quantification of impurities	5. Mutagenicity/genotoxicity
6. Solubility	6. Carcinogenicity
7. Partition coefficient (Log Po/w)	7. Reproductive toxicity
8. Further chemical and physical specifications	8. Acute toxicity
9. Homogeneity and stability	9. Repeated dose toxicity
10. Function and application	

A major challenge in evaluating potential health risks posed by tattoo inks is the assessment of exposure toward a certain chemical. Degradation products as a consequence of sun exposure or enzymatic activity may result in continuous release of chemicals into the blood circulation. Although considered inert, pigments present in the inks have a broad particle size distribution, falling also in the nanometer range (Schreiver et al. 2017). Hence, these aspects must be considered when assessing protective measures.

According to the General Product Safety Directive (GPSD) (Directive 2001/95/EC), tattoo ink manufacturers and distributors are responsible for their safety. As guidance for the safety evaluation procedure, numerous protocols have been compiled (BfR 2012; EDQM 2017). Yet, the main challenge in the application of standardized methods such as the OECD (Organisation for Economic Co-operation and Development) guidelines is the compatibility of these methods with insoluble substances such as pigments used in tattoo inks. The required endpoints as well as the specifications are summarized in Table 1. These can be divided into physicochemical characterization and toxicological evaluation.

A comprehensive physicochemical characterization of tattoo inks is inevitable for the evaluation of their safety. Beyond the chemical identity of the intentionally added pigments and auxiliary substances, impurities as well as degradation products shall be thoroughly identified and quantified. Nonetheless, results might differ depending on the sample preparation procedure. For example, the extraction of impurities from the pigments may vary in efficacy or trigger their degradation.

A battery of toxicological analysis adopted for the intradermal application route should be carefully considered for the ability of achieving conclusive results. Obstacles for the implementation of the tests guidelines are expected to appear in the following aspects:

1. Given the complexity of the ink mixture, the experimental design should describe whether a single substance or the entire mixture is being analytically addressed.

2. When performing in vitro assays, colorimetric measurements might not be suitable due to the broad light absorption spectra of the pigments used.
3. The applied dose of the test material may be compromised due to the sedimentation of the insoluble pigment particles.
4. The adjustment of the recommended guidelines is needed to assess possible toxic effects upon UV irradiation for simulating solar exposure or the removal of the tattoo with a laser. The phototoxicity may result in reduced viability of cells, causing subsequent photo-sensitization or genotoxicity. However, at present no methods exist which supply reliable information on the phototoxicity of tattoo inks.
5. For certain endpoints, in vivo testing might be unavoidable. For instance, the sensitizing or irritation nature of substances may be expressed or enhanced when combined with the trauma induced during the tattooing procedure. Moreover, in vivo tests may be triggered due to positive in vitro assays. The toxicological evaluation in living animals becomes even more required when assessing the systemic distribution of the substances, their accumulation in organs, as well as excretion kinetics. Although attempts have been made to envisage the biodistribution of pigments in mice, substantial and representative information is missing. In particular, for the extrapolation ability, mini pigs represent a much more suitable model relatively similar to human anatomy and physiology (Bode et al. 2010; Sullivan et al. 2001). Nevertheless, the OECD Test Guidelines are to date not validated for the intradermal application route.

Given the application route and the complex tattoo ink composition, the regulatory approach to ban the use of chemicals with known hazardous properties seems comprehensible. Based on a dossier compiled by the European Chemicals Agency (ECHA), the European Commission (EC) has published the first Union-wide restriction of substances used in tattoo inks and PMU (ECHA 2019). Briefly, according to the Regulation No. 1272/2008 of the European Parliament and of the Council, substances with harmonized classifications of hazard classes for carcinogenicity, mutagenicity, or toxicity to reproduction category 1A, 1B or 2; skin sensitization category 1, 1A, or 1B; skin corrosion category 1, 1A, 1B, or 1C or skin irritation category 2; or serious eye damage category 1 or eye irritation category 2 are subject to this restriction. Furthermore, substances restricted for use in cosmetic products according to the Regulation No. 1223/2009 shall not be used in tattoo and PMU inks. The rationale of this link to the cosmetic regulation is that substances, which were assessed as being unsafe when applied on top of the skin, shall not be injected into the skin. Further groups of restricted chemicals comprise heavy metals and certain pigments bearing an “azo” bond, which may result in cleavage and the release of carcinogenic PAAs.

The restricted substances shall not be present in tattoo inks and PMU above a certain concentration limit. Such concentration limits were established considering the minimization of health risks as well as the technically achievable detection limits of the substances. The utilized approaches for the assessment of risks posed by ingredients of tattoo inks and PMU are depicted in Fig. 2. Substances

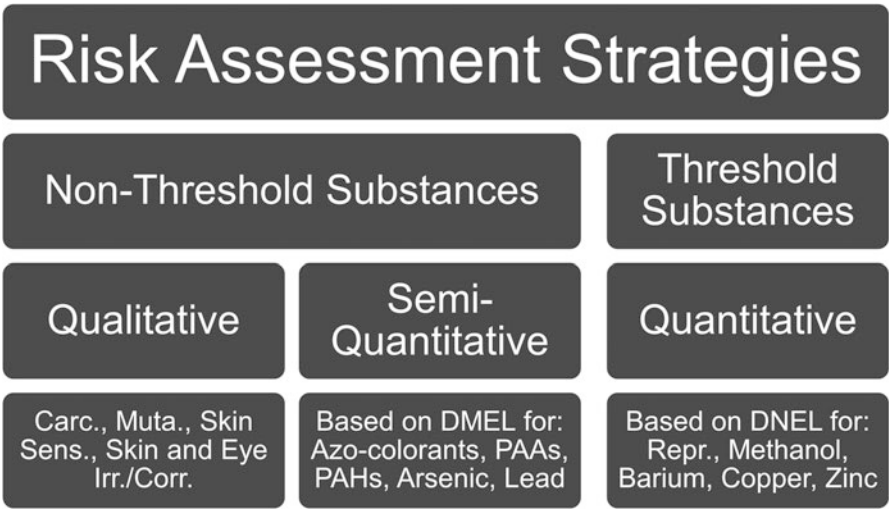

Fig. 2 Schematic representation of risk assessment strategies for the different groups of compounds

with no threshold hazard were evaluated in a qualitative manner. The qualitative assessment of risks is based on the fact that no concentrations could be determined at which the hazardous potency would be eliminated. In other words, for substances potentially causing carcinogenicity, mutagenicity, skin sensitization, and skin and eye irritation/corrosion, no dose-response correlations could be established due to missing data in general and missing assessments for the intradermal application route in particular.

For another group of non-threshold substances, concentration limits were set by applying the derived minimum effect level (DMEL). This approach may be used when adequate animal data is available to resolve a low concern of lifetime cancer risk (ECHA 2012). In this manner, substances present in tattoo inks, which pose a concern to human health, were treated. These include colorants of "azo" nature which may decompose by hydrolysis, enzymatic degradation or by UV irradiation into PAAs. Despite the low solubility of azo colorants, the systemic availability of dissolved molecules cannot be excluded. Moreover, PAAs may be present in azo colorants as impurities from the production stage. Hence, a representative DMEL value for *o*-anisidine was used to set a concentration limit for PAAs of concern using the endpoint carcinogenicity. For other substances of concern present in tattoo inks, e.g., impurities such as PAHs or heavy metals, DMELs for the most critical effect were used to set concentration limits.

Finally, a quantitative evaluation of risks could be performed for a group of substances by deriving their corresponding Derived No-Effect Level (DNEL). In this manner, reprotoxic substances, methanol, as well as certain heavy metals such as barium, copper, and zinc could be assessed. For example, a representative DNEL based on reprotoxic effects was derived for dibutyl phthalate, which was previously

detected in tattoo inks. In addition to assessment factors due to inter- and intraspecies differences, a factor due to possible cumulative effects was applied, resulting in a DNEL of 0.0007 mg/kg bw/day. The DNEL for barium compounds used as a common additive in tattoo inks could be established after applying correction factors for their intradermal injection. This value was derived for soluble compounds, assuming 100% absorption for oral studied where only 7% of the compound was absorbed after a single dose. In contrast, due to a very low solubility of barium sulfate, no release of ions is expected.

The concentration limits were derived subsequently to risk characterization. As an exemplary procedure for a quantitative assessment, the deviation of a concentration limit for reprotoxic substances of category 1A/B is described below. For the estimation of the exposure, mean parameters for the tattoo size, 300 cm^2, and the amount of injected ink, 14.36 mg ink/cm^2, for an average body weight (bw) of 60 kg were calculated to be 72 mg/kg bw/day (Engel et al. 2008; Laux et al. 2016; Prior 2015). The probability of occurrence of adverse effects for the defined exposure is expressed by the risk characterization ratio (RCR), which is the division product of the exposure estimate and the derived DNEL. Finally, the concentration limit for a certain substance in the ink is calculated by the reciprocal RCR for the substance of concern.

$$\text{Concentration limit} = \frac{\text{DNEL substance}}{\text{Amount of ink injected}}$$

Hence, based on the derived DNEL for dibutyl phthalate (0.0007 $mg_{substance}$/kg bw/day) and the calculated mean amount of tattoo ink injected (72 mg_{ink}/kg bw/day), the concentration limit was rounded to be 10 $mg_{substance}$ / kg_{ink}, which are 10 ppm or 0.001%.

It is important to mention that the parameters used for the exposure assessment depict a rather worst-case scenario in terms of the tattoo size, the repetitive exposure, as well as the bioavailability of the pigments and the released impurities. For instance, soluble substances were treated in a similar manner as non-soluble ones while assuming a complete release of impurities imbedded within the pigment particles. Yet, given the uncertainties, these parameters were considered as an appropriate estimation for reduction of health risks. Such uncertainties include the very limited knowledge on the toxicokinetics of substances injected into the skin in terms of chemical persistence or particle degradation into smaller fragments. The uncertainties become even greater when assessing the risks which may arise by nanoparticles (NPs). Numerous analyses of tattoo inks and tattooed skin have demonstrated the presence of particles in the nanometer size range (Høgsberg et al. 2011; Schreiver et al. 2017). However, differing from toxicological mechanisms described for distinct chemicals, NPs may trigger formation of reactive oxygen species, facilitate photochemical reactions, or cause immunoreactions as a result of particle overload. Parameters such as shape, size or crystallinity vary strongly for pigment particles and challenge the assessment of their toxic potential. For these reasons, the considerations of the worst-case scenario seem to comprise a rational measure for risk minimization.

Conclusion

Assuming a continuous increase in the tattooed population worldwide for the next decades, it seems that the first and foremost task of consumer protection authorities remains the clarification of risks associated with the tattooing procedure. Despite many uncertainties, much progress has been achieved so far. These findings served an important milestone for regulatory measures in the EU. Moreover, they allowed the clarification of possible adverse effects which might help individuals deciding for or against receiving a tattoo. Avoiding known allergens and keeping basic hygienic rules shall certainly decrease the likelihood of developing adverse reactions.

Cross-References

- ▶ Assessment of Mixtures: Bridging Principles and Other Approaches
- ▶ Default Factors in Quantitative Risk Assessment
- ▶ Nanoparticles and Their Regulation
- ▶ Toxicological Risk Assessment

Disclosure The authors declare that they do not have potential conflicts of interest.

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Risk Assessment of Food Additives

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Rainer Gürtler

Contents

Abstract

Food additives are substances used for technological purposes, such as preservation, sweetening, or coloring, during the production of food. The requirements for the risk assessment of food additives are described in guidance documents of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA). According to the EFSA guidance, a tiered approach which balances toxicity data requirements against the risk is applicable for the risk assessment of food additives. The approach was established to evaluate the following core areas: toxicokinetics, genotoxicity, toxicity (encompassing subchronic toxicity, chronic toxicity, and carcinogenicity), and reproductive and developmental toxicity. Additional studies on other toxicological endpoints may be required on a case-by-case basis. The approach

R. Gürtler (✉)
Department of Food Safety, Federal Institute for Risk Assessment (BfR), Berlin, Germany
e-mail: Rainer.Guertler@bfr.bund.de

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consists of three tiers. It provides guidance to applicants in designing their toxicity testing strategy in which the decision on the requirement for further testing can be based on the results of certain initial studies. While maintaining the high level of consumer safety, the application of this tiered approach could result in a smaller number of animal tests or more refined animal studies, compared to the requirements applied before, and thus benefit animal welfare. This chapter provides the legal background and delineates the principles and requirements for the risk assessment of food additives based on the guidance documents for food additive evaluations established by JECFA and EFSA.

Keywords

Food additives · Risk assessment · Tiered approach · Acceptable daily intake

Introduction

The use of chemical substances in order to maintain the quality of food has been a common procedure for a very long time. Accordingly, questions about the safety of these substances were addressed already many years ago. In 1955, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) of the United Nations established the Joint FAO/WHO Expert Committee on Food Additives (JECFA). In its first report, JECFA provided general principles for the use of food additives and pointed out that "critically designed animal tests of the physiological, pharmacological and biochemical behavior of a proposed additive can provide a reasonable basis for evaluating the safety of use of a food additive at a specified level of intake" (WHO 1957). Procedures for the testing of food additives were published by JECFA in 1958 and 1987 (WHO 1958, 1987). Currently, JECFA performs risk assessment of food additives based on principles and methods for the risk assessment of chemicals in food that were published in 2009 (WHO and FAO 2009). Since then, FAO and WHO have started several projects to update (sub) chapters (https://www.who.int/publications-detail/principles-and-methods-for-the-risk-assessment-of-chemicals-in-food (accessed on 26 May 2020)).

At the European level, the Scientific Committee for Food (SCF) was established by the Commission of the European Communities in 1974. The SCF evaluated food additives and advised the European Commission. In the course of re-organization of scientific committees, the SCF was re-named in 1997 into Scientific Committee on Food (SCF). The SCF was active until the European Food Safety Authority (EFSA) was established in 2003. Since then food additives have been evaluated by the respective EFSA Panels (Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC Panel) until 2008, Panel on Food Additives and Nutrient Sources Added to Food (ANS Panel) from 2008 to 2018, and Panel on Food Additives and Flavourings (FAF Panel) since 2018).

Any substances added intentionally to food including flavoring substances and processing aids might be considered as food additives; however, according to

European legislation, the term *food additive* is restricted to those substances which are used for technological purposes only. According to Article 3 of Regulation (EC) No. 1333/2008, a food additive "shall mean any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food, whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods." According to Article 6 of Regulation (EC) No. 1333/2008, a permission for the use of food additives can only be given for a substance provided that "(a) it does not, on the basis of the scientific evidence available, pose a safety concern to the health of the consumer at the level of use proposed; (b) there is a reasonable technological need that cannot be achieved by other economically and technologically practicable means; and (c) its use does not mislead the consumer." Accordingly, the safety of food additives is to be assessed by the EFSA prior consideration by the European Commission for market authorization. Applicants seeking such an authorization are required to provide all relevant data.

At a global level, based on risk assessments performed by JECFA, food additive standards were established by the Codex Committee on Food Additives (CCFA) of the Codex Alimentarius Commission (CAC) in the framework of the *Joint FAO/WHO food standards program*. In this respect, the CCFA established a General Standard for Food Additives (GSFA) with provisions for the use of food additives in certain food categories (http://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/gsfa/en/ (accessed on 26 May 2020)). The GSFA is not legally binding in the European Union. It may, however, be relevant for trade issues between the European Union and third countries if such cases were to be negotiated at the World Trade Organization (WTO).

Data Required for the Risk Assessment

The data required by JECFA for risk assessment of food additives are described in calls for data for each JECFA meeting (https://www.who.int/foodsafety/call-data/en/ (accessed on 26 May 2020)). These requirements are based on principles and methods for the risk assessment of chemicals in food (WHO and FAO 2009) and include chemical and technical considerations, biochemical aspects, toxicological studies, and observations in humans as well as data for exposure estimations. Specific guidance on the evaluation of and data requirements for food additives are also available from FAO (http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/guidelines0/en/ (accessed on 26 May 2020)) and WHO (http://www.who.int/foodsafety/chem/jecfa/guidelines/en/index.html (accessed on 26 May 2020)). According to these guidance documents, information from five main categories of toxicity studies on food additives should be routinely included: acute toxicity, short-term studies of toxicity, long-term

studies of toxicity and carcinogenicity, genotoxicity, and reproductive and developmental toxicity. Based on results obtained in these routine studies, there may be a need to perform special studies in order to investigate particular target organs, tissues, or endpoints.

The data required by EFSA for the risk assessment of food additives are described in a guidance document adopted by the ANS Panel in 2012 (EFSA 2012a). This guidance replaced the guidance established in 2001 by the European Commission's former SCF (2001) on which EFSA's AFC and ANS Panels based their risk assessments previously. According to the guidance from 2012, a tiered approach which balances toxicity data requirements against the risk is applicable for the risk assessment of food additives. The approach was established to evaluate the following core areas: toxicokinetics, genotoxicity, toxicity (encompassing subchronic toxicity, chronic toxicity, and carcinogenicity), and reproductive and developmental toxicity. Additional studies on other toxicological endpoints may be required on a case-by-case basis. The approach consists of three tiers. It provides guidance to applicants in designing their toxicity testing strategy in which the decision on the requirement for further testing can be based on the results of certain initial studies. While maintaining the high level of consumer safety, the application of this tiered approach could result in a smaller number of animal tests or more refined animal studies, compared to the requirements applied before, and thus benefit animal welfare.

According to EFSA's guidance for submission of food additive applications, a minimum set of data is required for all food additives at Tier 1. It covers data on absorption (i.e., systemic availability), genotoxicity in vitro, and subchronic toxicity. Depending on the results, further toxicity studies are required at Tier 2 and Tier 3. Tier 2 studies will be required in order to generate more extensive data for substances which are absorbed or demonstrate toxicity or genotoxicity in Tier 1 tests. If higher tier testing is required based on results in one of the core areas, such testing would only be required in this core area but not in the others, e.g., where results from the subchronic toxicity study require further Tier 2 studies but Tier 1 in vitro genotoxicity is clearly negative, there would be no need for Tier 2 follow-up of genotoxicity. Tier 3 testing should be performed on a case-by-case basis. Results at higher tiers will in principle supersede results observed at lower tiers.

Chemical Aspects and Manufacturing

Chemical data, including data on identity, chemical composition, purity, and stability of the food additives, are pivotal for reliable risk assessments. Requirements are described in Chap. 3 of the FAO and WHO guidance *Principles and Methods for the Risk Assessment of Chemicals in Food* (WHO and FAO 2009) and in the EFSA *guidance for submission for food additive evaluations* (EFSA 2012a). An example for the importance of adequate specification is titanium dioxide for which

the EFSA ANS Panel recommended in its scientific opinion on the re-evaluation of this food additive that the EU specifications for titanium dioxide (E 171) should include a characterization of particle size distribution using appropriate statistical descriptors (e.g., range, median, quartiles) as well as the percentage (in number and by mass) of particles in the nanoscale (with at least one dimension <100 nm) present in titanium dioxide (E 171) used as a food additive (EFSA 2016). The EFSA FAF Panel which is meanwhile responsible for the evaluation of food additives suggested an amendment for the current specification of titanium dioxide and recommended that, based on the proposed change in the specifications, revisiting the toxicological database on titanium dioxide (E 171) as a food additive should consequently be conducted in line with the data requirements specified in the EFSA guidance on nanotechnology (EFSA 2018, 2019a).

Information on the manufacturing of a food additive could provide useful indication for potential presence of impurities or unintended by-products that may require further consideration.

Toxicokinetics

The aim of investigations on systemic availability is to establish whether the substance or its breakdown products are absorbed from the gastrointestinal tract. In this respect the physico-chemical properties (e.g., molecular weight, hydro- and lipophilicity) of the substance should be considered as well as models for bioavailability from in vitro and in vivo studies. Demonstration of negligible absorption, either through experimental studies or based on theoretical considerations, may be considered as a scientific justification for not undertaking higher tiered toxicological studies provided that the results of the genotoxicity tests are clearly negative and no toxicity in the subchronic toxicity study is observed at Tier 1. Absorption data available for structurally related substances may contribute useful information. However, the required sensitivity to determine negligible absorption levels will generally necessitate in vivo studies using labelled compounds. If the absorption cannot be considered negligible at Tier 1, further data on absorption, distribution, metabolism, and excretion (ADME) including identification and quantification of metabolites are required at Tier 2. Basic single-dose toxicokinetic parameters, e.g., area under the curve of plasma concentration of the compound against time after oral administration, maximum concentration, time to reach maximum concentration, elimination half-life, and bioavailability, should be determined based on in vivo studies according to the Organisation for Economic Co-operation and Development (OECD) Technical Guidance (TG) No. 417. A range of dose levels should be applied in order to examine the linearity of kinetic parameters and possible saturation. The trigger for Tier 3 studies would be limited or slow excretion or any other mechanism resulting in bioaccumulation. In such a case, studies with repeated doses in experimental animals or human kinetic data from volunteer studies may be required.

Genotoxicity

The in vitro investigations on genotoxicity at Tier 1 should cover gene mutations and structural and numerical chromosomal alterations as recommended by the EFSA Scientific Committee (EFSA 2011a). In line with this recommendation, a bacterial reverse mutation assay (OECD TG 471) and an in vitro mammalian cell micronucleus test (OECD TG 487) are required for all food additives. Any inconclusive, equivocal, or positive results observed with in vitro tests at Tier 1 should be further investigated. According to EFSA's guidance document on genotoxicity testing strategies (EFSA 2011a), inconclusive and equivocal test results may be clarified by further in vitro testing, but in vivo studies might also become necessary. A positive result in a Tier 1 study requires follow-up at Tier 2 in order to investigate whether the hazard is expressed in vivo. Suitable tests for a follow-up of results from Tier 1 studies are an in vivo micronucleus test (OECD TG 474), a transgenic rodent somatic and germ cell gene mutation assay (OECD TG 488), and an in vivo comet assay (OECD TG 489). Detailed advice on strategies for genotoxicity testing is given in an opinion of the EFSA Scientific Committee (EFSA 2011a). In the case of negative in vivo genotoxicity tests, it is crucial to demonstrate, based on cytotoxicity or kinetic data or other lines of evidence, that the target tissue was exposed. EFSA's Scientific Committee provided advice on the adequacy to demonstrate target tissue exposure in in vivo studies, particularly in the mammalian erythrocyte micronucleus test (EFSA 2017a). In case the food additive is a mixture of substances, the statement of EFSA's Scientific Committee on the genotoxicity testing of chemical mixtures (EFSA 2019b) should be consulted. The Scientific Committee was also asked to provide guidance on the most appropriate in vivo tests to follow up on positive in vitro results for aneugenicity and on the approach applicable for risk assessment of substances that exhibit aneugenicity but do not induce gene mutations or clastogenicity. A draft guidance on aneugenicity assessment was published for public consultation in March 2020 (http://www.efsa.europa.eu/en/consultations/call/public-consultation-draft-scientific-committee-guidance-assessment (accessed on 26 May 2020); after public consultation (deadline 31 May 2020), EFSA's websites may be consulted for the final version).

Genotoxicity in vivo is to be considered as an adverse effect per se, even in cases where cancer bioassays are negative, since genotoxicity is also implicated in diseases other than cancer and one of the aims for genotoxicity testing is to identify substances which could cause heritable damage in humans (EFSA 2011a). There is no Tier 3 for genotoxicity testing. If a substance is positive at Tier 2, it is usually assumed that it is a somatic cell genotoxin and will be potentially carcinogenic and also mutagenic in germ cells. Such substances are not considered acceptable as food additives. Hence careful consideration should be given to animal welfare before conducting any further in vivo studies (EFSA 2012a). It should, however, be noted that the assessment of genotoxicity is generally based on all available data and that the quality and reliability of data are taken into consideration (EFSA 2011a). Accordingly, it is important to differentiate between indication and clear evidence for genotoxicity in vivo. An indication for in vivo genotoxicity would require further clarification.

Subchronic and Chronic Toxicity and Carcinogenicity

A subchronic toxicity study should be performed for a period of at least 90 days in rodents (OECD TG 408) at Tier 1. The design of this study should be modified to include the assessment of some additional parameters described in the more recent guideline on repeated-dose 28-day oral toxicity studies in rodents (OECD TG 407). Toxicity observed in the subchronic toxicity study would trigger investigation of chronic toxicity at Tier 2. A chronic toxicity study may reveal effects which were not observed in subchronic studies, or it may confirm effects at the same or even lower doses than those applied in the subchronic study. Chronic toxicity and carcinogenicity are to be investigated at Tier 2 either separately (OECD TG 452 and 451) or in a combined study (OECD TG 453). The EFSA ANS Panel noted in its guidance document that there was a considerable debate in the area of risk assessment of pharmaceuticals about the value of the two rodent species approach for the evaluation of carcinogenicity and that this debate has led to the suggestion that there may be no need to continue investigating carcinogenicity routinely in two species (EFSA 2012a). The Panel supported this position and recommended, deviating from former requirements, to perform the studies with a single species only, generally the rat. However, under certain circumstances, e.g., indications for species-specific effects, a study in a second species may become necessary. At Tier 3, studies on the mode of action may be performed if required.

Reproductive and Developmental Toxicity

The subchronic toxicity study performed at Tier 1 provides only limited information on reproductive toxicity and no information on developmental toxicity. It provides information on potential effects on the reproductive organs and, if assessed, the estrous cycle, but it does not cover fertility and the whole reproductive cycle. However, the decision on whether studies on reproductive and developmental toxicity are required could be based on the outcome of the subchronic toxicity study provided that the absorption of the substance is negligible. Studies on reproductive and developmental toxicity will generally be required for substances which are systemically available. In addition, any indication for effects on reproductive organs or parameters in the subchronic toxicity study will trigger testing for reproductive and developmental toxicity at Tier 2. This comprises a prenatal developmental toxicity study (OECD TG 414) in rabbits and an extended one-generation reproduction toxicity study (EOGRTS) (OECD TG 443). According to the OECD Guideline 443, the EOGRTS covers parameters on reproductive endpoints, pre- and postnatal developmental endpoints, and specific endpoints on developmental neurotoxicity, immunotoxicity, and endocrine disruption. The EOGRTS protocol includes the assessment of parameters which can be used for the decision on whether assessment of a second generation is required. The risk assessment may be based on the results of these studies; however, the effects observed might trigger additional studies at Tier 3, e.g., on endocrine effects, developmental neurotoxicity (OECD TG 426), and mode of action (EFSA 2012a).

Other Studies

In addition to the core areas for evaluation, other studies may be required for the risk assessment of food additives, e.g., studies on immunotoxicity, hypersensitivity, and food intolerance. Likewise, human studies, e.g., ADME studies and tolerance studies, could provide useful information (EFSA 2012a). However, the quality and reliability of tolerance studies are mainly dependent on the study design. The prevalence of intolerances against food additives which are already on the market could reliably only be determined with placebo-controlled double-blind oral challenge tests, a condition which is met only by a few studies (Simon 2003; Wilson and Bahna 2005; Skypala et al. 2015).

Several symptoms have been attributed to food additive exposure, but the cause-and-effect relationship has not been well demonstrated in all (Wilson and Bahna 2005; Turner and Kemp 2012; Bahna and Burkhardt 2018). Allergenicity may result from the consumption of food additives which are proteins or peptides, e.g., lysozyme (E 1105) and invertase (E 1103), while pseudoallergenicity could be due to other (non-protein) food additives. The EFSA ANS Panel noted in its guidance document (EFSA 2012a) that there are no validated studies in laboratory animals which would allow assessment of the potential of a substance to cause allergic reactions in susceptible individuals following oral exposure. The panel recommended to consult the EFSA guidance on the allergenicity of genetically modified organisms (EFSA 2010) if the additive is a potential allergen, e.g., a protein or a peptide, or contains residues of proteins or other known allergenic molecules (EFSA 2012a). However, the panel pointed out that defining a threshold or a no observed adverse effect level (NOAEL) is difficult and that, accordingly, an adverse effect would be taken into account on a case-by-case basis (EFSA 2012a).

For new food additives, an indication for immunotoxicity may be obtained from the studies performed at Tier 1 and Tier 2. The subchronic toxicity study in rats (OECD TG 408) performed at Tier 1 involves investigation of a number of parameters that may be indicative of an immunotoxic or immunomodulatory effect, e.g., changes in spleen and thymus weights relative to body weight in the absence of overt toxicity, histopathological changes in these and other organs of the immune system, as well as changes in total serum protein, albumin/globulin ratio, and the hematological profile of the animals. Such effects may be confirmed or, alternatively, observed for the first time in Tier 2 studies, notably the EOGRTS (OECD TG 443), but also in chronic toxicity/carcinogenicity studies conducted according to OECD TGs 452, 451, or 453. In the EOGRTS, a cohort of animals is specifically dedicated to assess the potential impact of exposure on the developing immune system. If the results from these studies provide indication for immunotoxicity, additional studies may be performed at Tier 3 in order to investigate the underlying mechanisms of the effects seen and to assess their relevance for the risk assessment (EFSA 2012a). The EFSA ANS Panel noted in its guidance document that there are no OECD guidelines for such extended specialized studies and recommended to consult a WHO guidance for

immunotoxicity risk assessment for chemicals (WHO 2012). The respective sections on immunotoxicity and food allergy and other food hypersensitivities of the *Principles and Methods for the Risk Assessment of Chemicals in Food* (WHO and FAO 2009) may also be consulted.

Derivation of an Acceptable Daily Intake

A main purpose of the risk assessment of food additives is the derivation of an Acceptable Daily Intake (ADI) as a health-based guidance value. An ADI is the estimated maximum amount to which individuals may be exposed daily over their lifetimes without appreciable health risk. Based on the most sensitive endpoint from a range of toxicological hazards and their dose-response relationships, a No Observed Adverse Effect Level (NOAEL) or, preferably, a benchmark dose lower confidence limit (BMDL) is established and used as point of departure for deriving an ADI (EFSA 2012a, 2017b). This point of departure is divided by an uncertainty factor which covers uncertainties due to the extrapolation of data from animal studies to the human situation as well as individual variabilities. Generally, a factor of 100 is applied as a default value. A smaller factor could be applied if human data are available, e.g., toxicokinetic data, which allow for comparison of internal doses in experimental animals and humans. A factor larger than 100 would be applicable if additional uncertainties were to be covered. The ADI is expressed in mg per kg body weight and is established for compounds for which a threshold mechanism of toxicity can be demonstrated. The ADI is applicable to the general population except infants below 16 weeks (WHO 1978; SCF 1998; EFSA 2017c). The EFSA Scientific Committee provided guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age (EFSA 2017c).

The EFSA ANS Panel (EFSA 2014) presented a conceptual framework for the risk assessment of certain food additives

- which are of low intrinsic toxicity, e.g., substances with Acceptable Daily Intake (ADI) "not specified" (no numerical ADI),
- which are authorized in food categories according to *quantum satis* (QS) (Regulation (EC) No 1333/20084) which precludes a reliable exposure estimate,
- which, while they are not of low intrinsic toxicity, are of low toxicological concern as used in food, e.g., sodium hydroxide.

According to this conceptual framework, the outcome of the risk assessment, taking into account all available information, depends on the availability of reliable information on both exposure and toxicity and on whether or not the food additive and/or its breakdown products/metabolites are identical to a compound which is a normal constituent in the body (an endogenous compound) and/or is a regular component of the diet.

Exposure Assessment

For exposure assessment of food additives, a tiered approach is recommended by FAO and WHO (WHO and FAO 2009) and is used by JECFA for the evaluation of food additives. The initial steps rely on conservative screening methods such as the budget method or models based on poundage data.

EFSA developed a Food Additives Intake Model (FAIM) template to support the calculation of exposure estimates to food additives. An updated FAIM version is available at https://www.efsa.europa.eu/en/applications/foodingredients/tools (accessed on 26 May 2020). This FAIM template is a screening tool for estimating long-term exposure to food additives. It can be used as a first step in the dietary exposure assessment by applicants, risk assessors, and risk managers. It is based on summary food consumption statistics (in grams per kg body weight) from the EFSA Comprehensive European Food Consumption Database tailored to food additive categories. Based on FAIM, the mean and high-level exposure to food additives can be estimated for different population groups throughout several European countries and age groups. According to the "Guidance for submission for food additive evaluations" (EFSA 2012a), two scenarios can be applied, taking into account:

- The maximum permitted levels (MPLs) of use, as set in the current EU legislation.
- The levels of use as reported for existing food additives, if available, or as proposed, in the case of new applications.

The model also provides information on the food groups contributing to the total mean exposure. The FAIM template can be used for the estimation of exposure to a new food additive or exposure resulting from new uses of an already authorized food additive.

More refined methods should be applied when screening methods cannot rule out a safety concern, e.g., when the exposure estimated based on FAIM exceeds the ADI. Data from the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) (EFSA 2011b) can be used for refined exposure assessment. A guidance for the use of the Comprehensive Database is available at http://www.efsa.europa.eu/en/food-consumption/comprehensive-database (accessed on 26 May 2020). This database is used by EFSA to estimate exposure for the six following population groups: infants (from 12 weeks of age), toddlers, children, adolescents, adults, and the elderly. Dietary exposure is in the majority of cases estimated based on consumption data for all foods and all subjects involved in the dietary surveys, but estimates for "consumers only" are also calculated under specific circumstances and brand loyalty is considered by EFSA if appropriate (Gürtler and Arcella 2020).

In addition to food consumption data, the concentration of the additive in food as consumed is an important aspect for the exposure assessment. Depending on the purpose of the exposure assessment (pre-regulation or post-regulation), food additive concentration data can originate from:

- Legislated maximum permitted levels (MPLs)
- Usage levels reported by manufacturers
- Monitoring and surveillance programs
- Total Diet Study (TDS).

More details on refined exposure assessments for food additives, including recommendations of international expert committees, uncertainties and challenges related to the exposure estimation for food additives, and the monitoring of food additive intake, are described elsewhere (Gürtler and Arcella 2020).

Risk Management Based on Risk Assessments

Based on a numerical ADI and an exposure assessment, conditions of use, e.g., maximum level for certain food categories, can be derived for food additives by risk managers. The current conditions of use applicable in the European Union are defined in Regulation (EC) No 1333/2008 and its amendments. For some food additives, there are no numerical ADIs. For substances of very low toxicity which, on the basis of the available data on toxicity and intake, do not represent a hazard to health, e.g., citric acid and citrates (E 330–E 333), the outcome of the evaluation by JECFA or SCF was "ADI not specified." Such substances may be used at "*quantum satis*" level. According to Regulation (EC) No. 1333/2008, "'*quantum satis*' shall mean that no maximum numerical level is specified and substances shall be used in accordance with good manufacturing practice, at a level not higher than is necessary to achieve the intended purpose and provided the consumer is not misled."

At a global level, the Codex Alimentarius Commission with its Codex Committee on Food Additives (CCFA) is responsible for risk management measures for food additives based on risk assessments carried out by JECFA. CCFA established a General Standard for Food Additives (GSFA) (http://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/gsfa/en/ (accessed on 26 May 2020)) with provisions for the use of food additives in certain food categories.

An occasional exceedance of the ADI might be tolerated provided that (i) there is still a tolerable margin of safety between the NOAEL and the exposure, (ii) the effect on which the ADI was derived is not observed after acute exposure, and (iii) the exceedance does not occur frequently so that the long-term exposure is not significantly affected (Gürtler 2010). However, the provisions of use should ensure that the ADI is generally not exceeded. Therefore, exposure assessments need to take into account not only the reported uses but also the provisions of use, e.g., the maximum levels for certain food categories.

For compounds for which no safe level of exposure can be anticipated, for example, genotoxic carcinogens, an ADI would not be established. Such substances would not be acceptable as food additives. For the assessment of the risk resulting from levels of unavoidable contaminants or residuals in the additive which are genotoxic and carcinogenic, the EFSA FAF Panel generally uses the margin of exposure (MOE) approach described in an EFSA Scientific Committee opinion

(EFSA 2005, 2012b). The Threshold of Toxicological Concern (TTC) approach would be considered for the evaluation of unavoidable genotoxic residuals, for which carcinogenicity data are not available. In such cases exposures for high-level consumers at the proposed maximum use levels would be expected to be below the TTC for potential DNA-reactive mutagens and/or carcinogens of 0.15 μg/person/day (EFSA 2012c, 2019c). The TTC approach could also be applied to low-exposure substances such as impurities, metabolites, and degradation products of deliberately added substances for which genotoxicity data may be unavailable (EFSA 2012a).

In 2010, a re-evaluation program was established in the European Union for all existing food additives. According to Regulation (EU) No. 257/2010, food additives which were permitted before 20 January 2009 shall be subject to a new risk assessment carried out by EFSA. In order to prioritize the evaluation of more than 300 food additives, priority criteria such as time since the last evaluation by SCF or EFSA and the availability of new scientific evidence and exposure were applied (http://www.efsa.europa.eu/en/topics/topic/food-additive-re-evaluations (accessed on 26 May 2020)). The highest priority was assigned to certain food colors for which the evaluations were to be completed until the end of 2010, while the lowest priority was applied to sweeteners, except aspartame, which should be evaluated until the end of 2020.

Perspective

There are several challenges related to the exposure estimation for food additives and the monitoring of food additive intake, including challenges related to classification of food into food categories and challenges related to analytical methods (Gürtler and Arcella 2020). The links between food consumption information on the one hand and food additive concentration data on the other are mostly not compatible with each other due to inconsistent food classification systems. Food categories, as defined in legislation, e.g., Regulation (EC) No 1333/2008, for which in many cases food additive concentration data are available, are not fully identical with the food categories for which food consumption data are available. It is therefore a challenge to reduce the resulting uncertainties, e.g., by harmonization of such classification systems. Another challenge is related to the monitoring of the use of food additives. Validated analytical methods are needed to fulfill the monitoring requirements according to Regulation (EC) No 1333/2008. However, whereas there is a European reference laboratory for feed additives, no reference laboratory exists for food additives. Thus, it is still a challenge to harmonize the analytical methods that are required for the monitoring of food additives.

The current EFSA guidance document for food additive evaluations (EFSA 2012a) provides a flexible approach which acknowledges the use of integrated testing strategies and alternative methods in order to complement the data required in this guidance. In some cases, e.g., for the evaluation of aspartame, physiologically based pharmacokinetic modelling was applied, and it may be expected that such

modelling will become more relevant for the evaluation of additives in future. "In silico−/(quantitative) structure activity relationships (QSAR)" methods may contribute to evaluate impurities and metabolites and may also be used for screening purposes before embarking on any Tier 1 testing. Special studies may be used to investigate the mode of action in the tiered approach as recommended by EFSA (2012a). Investigation of adverse outcome pathways (AOPs) might provide useful information that may support risk assessments for food additives in future. In an analysis performed by Kramer et al. (2019), the adverse effects and related points of departure based on which an ADI was derived by EFSA, SCF, or JECFA were compiled for all food additives permitted in the European Union. According to this study, a point of departure for adverse effects was identified by EFSA/SCF/JECFA only for 31% of the 326 food additives considered. Adverse effects were mainly observed in the liver, kidney, cardiovascular system, lymphatic system, central nervous system, and reproductive system. Several AOPs related to these endpoints were identified by Kramer et al. (2019) in an OECD compilation of AOPs (https://aopkb.oecd.org/ (accessed on 26 May 2020)). However, the level of development and documentation was different for these AOPs, and there is still a lack of AOPs for several adverse effects such as gastrointestinal tract irritation and a number of non-specific, late-onset, and/or local effects, including body weight changes (Kramer et al. 2019; Vinken et al. 2020). Kramer et al. (2019) pointed out that AOPs cannot be used as stand-alone tools. In combination with other relevant data, AOPs might, however, be used as supporting information for risk assessments for food additives in future. According to Vinken et al. (2020), a wider role for AOPs in future risk assessment may probably best focus on providing support for read-across; however, a number of challenges need to be tackled.

Cross-References

- ▶ Novel and Traditional Foods: Novel Food Regulation in the EU
- ▶ Registration and Approval in Regulatory Toxicology
- ▶ Risk Assessment of Food Components with Botanical Origin
- ▶ Risk Assessment of Genetically Modified Food and Feed
- ▶ Toxicological Risk Assessment in Different Jurisdictions

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Risk Assessment of Food Components with Botanical Origin

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Birgit Dusemund

Contents

Abstract

In view of a persisting trend to use botanicals and botanical preparations especially in food supplements but also in replacing synthetic additives or flavorings in food, aspects of the safety evaluations of plants and plant-derived components in food are discussed. In addition risk assessment regarding unintentional intake of botanical ingredients via contamination of food with whole plants, plant parts, or their components is addressed. Examples are presented taking the complexity in composition of botanicals and their matrix effects into account. Requirements and principles of present guidelines for the safety evaluation of botanicals and their components for food use, including a presumption of safety approach based on existing knowledge, are outlined. The essentials of relevant regulatory frameworks are summarized, and an outlook on possible future developments is given.

B. Dusemund (✉)
Department of Food Safety, Federal Institute for Risk Assessment (BfR), Berlin, Germany
e-mail: Birgit.Dusemund@bfr.bund.de

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Botanicals in Food: An Introduction

Plant-derived food forms an intrinsic part of traditional diets all over the world. Furthermore, diets rich of vegetables and fruits are associated with health benefits. As part of a continuing trend to a preference for natural, "organic," *herbal*, and plant-based products, especially *food supplements* (synonym: *dietary supplements*) based on *botanicals*[1] and their preparations,[2] became widely available on the European and North American market. This was accompanied by the tendency to replace synthetic food additives or flavorings in food products by those of botanical origin. Novel foods or genetically modified foods represent another source of intentional dietary intake of botanicals and are not dealt with here (see ▶ Chap. 93, "Risk Assessment of Genetically Modified Food and Feed"). Unintentional exposure of consumers to botanicals and their components is occurring via *contamination* of food with whole plants, plant parts, or their ingredients, e.g., as a consequence of inadequate harvesting or cleaning methods, adulteration or due to carry over from animal feed through meat or animal products, or via mistaking edible and nonedible plants (Speijers et al. 2010; Koleva et al. 2012; Dusemund et al. 2017a). The use and occurrence of plants and their components in feed are not a subject of this chapter.

The use of a large variety of botanicals in food supplements and related products includes that of certain medicinal plants. In particular plants used for therapeutical purposes may contain a diversity of biologically active substances such as alkaloids; cardiac glycosides; mono-, di-, tri-, and sesquiterpenes; and anthraquinones for which the induction of desired or adverse effects depends on dosage. As known from many lawsuits, the borderline between plant-based food supplements (not subjected to an authorization procedure in the European Union (EU)) and plant-based medicinal products (subjected to an authorization procedure in the EU) is often difficult to define. In relevant literature the need of an EU-wide harmonized approach based on appropriate systematical safety evaluations of botanicals and botanical preparations used in food supplements is described to assure that intake doses of biological active plant components in these products are within safe limits (EFSA 2009a, b; Silano et al. 2011; EFSA 2014).

Specifics in the Risk Assessment of Botanicals: Some Examples

Independent from the role of the botanical, being either added for sensory, technological, or health purposes to a food product or occurring as a natural typical component or an impurity in food, risk assessments of botanicals and their preparations differ from that of clearly defined single substances as a component of food. Thus, it has to be considered that botanicals generally consist of a *complex mixture of phytochemicals*. Regarding the active principle of interest for technological or health reasons, the purity is often rather low, and the remaining substances are not characterized sufficiently. A botanical preparation of a specific species may be represented by different extracts varying in composition, due to the use of different source materials (e.g., different botanical subspecies, different geographical origin and

conditions of growth and harvesting, different parts of a plant) and different extraction methods and solvents. In case of selective extractions, enrichments of natural ingredients of concern or contaminants such as heavy metals or pesticides may occur. Pharmacological and toxicological effects of botanical products are usually associated with their contents of secondary plant metabolites which can vary among plants belonging to the same species or variety as a consequence of multiple biotic and abiotic factors.

Identity and Specifications

The contents of secondary plant metabolites and the botanical origin have to be taken into account by defining the *identity and specifications* of botanical preparations which of course is also basically important to exclude adulteration and misidentification of plants. Furthermore it has to be ascertained in the risk assessment that specifications of the products of trade and those investigated in the toxicity testing are the same or allow read-across.

These reasonings may be illustrated by an example. For the natural botanical food color lutein (E 161b), belonging to the category of food additives, the content of total carotenoids/saponified carotenoids as coloring matter may be as low as 5% when extracted from grass, nettle, lucerne, or spinach but amounts to $\geq$60% when *Tagetes erecta* flowers are used as a source. While the EFSA (European Food Safety Authority) Panel on Food Additives and Nutrient Sources Added to Food (ANS) in the process of reevaluation of food additives could establish an ADI of 1 mg/kg body weight (bw)/day for the more concentrated extracts (total carotenoids $\geq$60%) from *Tagetes erecta* flowers, it decided that the database available is too limited to conclude that the ADI also applies to lutein preparations of lower purity or from other sources (EFSA 2010, 2011a).

Interactions of Secondary Plant Components

Furthermore, in a botanical biologically active substances usually occur accompanied by chemically related compounds formed, e.g., as precursors or by-products of biosynthesis pathways. Due to similar structures, a variety of components of a botanical or botanical preparation may have the same structural alerts for specific toxicity endpoints, may react as agonists or antagonists regarding receptor-mediated reactions, or may compete for binding sites in metabolizing enzymes responsible for activation, deactivation, or elimination. In consequence *interactions with accompanying ingredients* which may weaken or enhance the toxic effects of a known substance of concern have to be considered in the risk assessment of plant materials (*matrix effects*). Thus, in general, basing the risk assessment of botanicals, botanical preparations, or botanical contaminants on the exposure and toxicity data of only one active ingredient and ignoring the accompanying ones may be inappropriate. It also becomes evident that breeding methods, influencing biosynthesis pathways, may

change the pattern in which bioactive substances occur in a botanical and thus affect its toxicity profile. If botanical preparations of different plants are used in combinations, which is frequently observed, the possibilities of interactions increase.

Possible interactions including synergistic effects have been addressed, e.g., in the risk assessments of botanical contaminants in food and feed recently performed by national authorities and EFSA (e.g., Dusemund et al. 2017b). Evaluating the contamination of poppy seeds, derived from *Papaver somniferum*, by opium alkaloids, in addition to the presence of morphine that of codeine as a precursor in the biosynthesis of morphine, has been considered, both alkaloids acting as agonists binding to the μ-opioid receptors (BfR 2005; EFSA 2011b). An altered pattern of alkaloids was, e.g., observed in Australian poppy seeds originating from poppy cultivars developed by genetic regulation of certain enzymatic biosynthesis processes to give a high yield of thebaine and oripavine, two other intermediates in the biosynthesis of morphine, which are used as precursors in drug synthesis (EFSA 2011b). In the risk assessment of ergot alkaloids occurring in the sclerotia of *Claviceps purpurea*, which are contaminating grain, reference is made to a sum of ergot alkaloids including, i.a., ergometrine, ergotamine, ergosine, ergocristine, ergocryptine, and ergocornine. They have in common the tetracyclic ergoline ring system which is associated with their activity as ligands for adrenergic, serotonergic, and dopaminergic receptors (EFSA 2012a). Additive toxic effects have also been taken into account in the risk assessments of pyrrolizidine alkaloids, which have been performed for the contamination of salad with parts of *Senecio vulgaris* (BfR 2007), and for the occurrence of pyrrolizidine alkaloids in honey (BfR 2011a; EFSA 2011c), herbal teas, and other food commodities including food supplements (BfR 2013, 2016; EFSA 2017a). A cumulative assessment approach is recommended for all 1,2-unsaturated pyrrolizidine alkaloids, the double bond being a prerequisite for metabolic activation to genotoxic carcinogens (COT 2008; BfR 2016, EFSA 2017a). A group approach has also been chosen in the risk assessment of quinolizidine alkaloids, such as sparteine, lupanine, and lupinine, exhibiting anticholinergic activity and occurring in food prepared from seeds of bitter lupines (certain varieties of, e.g., *Lupinus albus*, *L. angustifolius*, *L. luteus*) without adequate debittering process (BfR 2017). Interactions may as well play a role regarding the toxicokinetic and toxicodynamic effects of polyphenols contained in green tea extracts derived from the leaves of *Camellia sinensis* which are inter alia used in food supplements. It has been hypothesized that the principal ingredient (−)-epigallocatechin-3- *O*-gallate (EGCG), taken as part of a green tea extract, shows slower elimination than when used as an isolated compound due to competition for binding sites in metabolizing enzymes with other accompanying polyphenols in the extract (EFSA 2009a).

Experiences from Human Exposure

The risk evaluation of botanicals which have been traditionally used for years as food or *herbal* medicine is often based on the *experiences and data from human exposure*, covering potentially epidemiological and clinical studies, reports of

adverse health effects, and case reports of intoxications, while data of validated toxicological studies in experimental animals are often sparse.

Furthermore, botanical ingredients that commonly occur at low levels in botanical components of the diet may be extracted and reintroduced in concentrated form in certain specific food supplements or related health products resulting *in comparatively high exposure levels* (EFSA 2004). There are some examples where partly even serious adverse effects have been assigned to this type of products. Thus, capsules containing high-dosed dried green tea extracts taken for weight-loss purposes have been associated with severe liver toxicity, while these adverse effects are not reported for traditional consumption of green tea infusions (EFSA 2009a; Speijers et al. 2010). Certain case reports on adverse cardiovascular effects are suspected of being associated with intake of food supplements advertised for weight loss or improvement of physical fitness containing high doses of (−)-synephrine as part of bitter orange (*Citrus aurantium* ssp. *aurantium*) extracts partly in combination with caffeine. No adverse effects are known from (−)-synephrine exposure via traditional foods such as orange juice or bitter orange marmalades (NTP/NIEHS 2004; EFSA 2009a; Health Canada 2011; BfR 2012a, Bakhyia et al. 2017).

Guidance for Safety Evaluation: An Approach by EFSA

In view of the described specific aspects in the safety evaluation of plant-based food supplements and their expanding market volume and increasing variety with numerous claims, EFSA saw a need for a better characterization of the botanicals in use and for a harmonization of their risk assessments (EFSA 2004). To identify the data needed to assess the safety of botanicals and to suggest a science-based approach for the safety assessment, EFSA developed the "*Guidance on safety assessment of botanicals* and botanical preparations intended for use as ingredients in food supplements" (EFSA 2009b). The guidance is intended to assist risk assessors and food manufacturers considering the safety of a given botanical. Even though the guidance is focussed on the use of botanicals in food supplements, EFSA emphasizes that the principles of the approach chosen are applicable also to other uses of botanicals and botanical preparations in the food and feed areas. This guidance does not refer to the use of botanicals or botanical preparations for use as a novel food or botanicals representing genetically modified food, since for both categories special guidances do exist (see ▶ Chap. 93, "Risk Assessment of Genetically Modified Food and Feed").

The information considered as necessary for a botanical or botanical preparation is *technical*, *exposure*, and *toxicological* data. The *technical* data comprise details on (i) the identity and nature of the source material, (ii) the manufacturing process of the botanical (preparation), (iii) its chemical composition, (iv) its specifications, (v) its stability in food (supplements), (vi) the proposed uses and use levels, and (vii) the information on existing assessments. Regarding *exposure* data information is required on (i) the anticipated exposure via the food supplement; (ii) the cumulative exposure via different categories of food, including food supplements, and medicinal

products; (iii) the modality of use; and (iv) the information on historical use of different categories of food, including food supplements, and medicinal products. Regarding the *toxicological* data, studies on toxicity and toxicokinetics including metabolism of botanicals and botanical preparations should be conducted using internationally agreed protocols.

A two-level tiered approach for the safety evaluation of the botanical (preparation) is proposed depending on the available knowledge. It consists primarily on *level A* of a risk assessment in which, based on all available data, a decision is derived if there is (i) a safety concern, (ii) no safety concern, or (iii) a need for additional data. In the latter case, the requirement for further testing is specified on a subsequent level (*level B*).

On *level A* the decision "no safety concern" may be based on the principle of a "presumption of safety." The guidance describes that a *presumption of safety* could be applied when available data would allow to conclude that exposure to known levels of the botanical (preparation) has occurred in large population groups for many years without reported adverse effects and that thus no additional data are judged necessary for the safety evaluation. Requirements for a "presumption of safety" are that not only use levels but also chemotypes of the botanicals and the chemical composition of the botanical preparations should be in line with historically used ones and intakes due to the intended levels of use are within the range of intake levels derived from the European Member States' average diets. The approach relies mainly on the objective of not significantly increasing exposures beyond the levels linked to the safe history of use.

If specific compounds of concern can be well defined on *level A*, the evaluations can focus on them. For a botanical (preparation) with a potential to contain toxic, addictive, psychotropic, or other substances that may be of concern, "presumption of safety" can be applied only if there is convincing evidence that these undesirable substances are either absent or significantly reduced or inactivated during processing. In these cases a "presumption of safety" of the botanical (preparation) is only justified when the overall exposure to the substances of concern is not too high compared to existing health-based guidance values such as the acceptable/tolerable daily intake (ADI/TDI). Consideration of exposure to the substance of concern in relation to the Threshold of Toxicological Concern (TTC) values may also be helpful. When the botanical (preparation) contains substances that are both genotoxic and carcinogenic, the "Margin of Exposure" (MOE) approach (EFSA 2005) could be applied. Furthermore, the EFSA guidance (EFSA 2009b) addresses the possibility that the kinetic and toxicodynamic of a naturally occurring substance could be modified by the matrix in which it is present which may result in reduced or increased toxicity. Advice is also given regarding read-across between two different preparations of a botanical or between different botanicals for which equivalence of composition data and consumption patterns regarding the substances of concern is a precondition.

On *level B* decision is taken which additional studies are needed for those botanicals or botanical preparations for which a "presumption of safety" was not justified on *level A* because, e.g., the anticipated intake is significantly higher than

the estimated historical intake level or the historical intake level cannot be assessed. According to the EFSA guidance (EFSA 2009b), the study requirements on *level B* can be deduced from the "Guidance on submissions for food additive evaluations by the Scientific Committee on Food (SCF 2001)."[3] The spectrum of toxicological data asked for comprises primarily studies on toxicokinetics including metabolism, genotoxicity, and subchronic toxicity. Depending on the outcome of these studies and other specific relevant information, further studies, e.g., on reproductive toxicity, developmental toxicity, neurotoxicity, immunotoxicity, and chronic toxicity/carcinogenicity, may be required. The specifications and identity criteria for the botanical preparation(s) used for the toxicity studies and their relationship to the final product to be used in the food supplement should be described in detail.

The adequacy of the two-level tiered approach described in the guidance document was tested with a selected number of examples including botanicals known to contain acute or subchronic toxic substances or potentially genotoxic carcinogens and botanicals with an established history of food use. The results of this study were published in a report illustrating how to apply the guidance document (EFSA 2009a). In 2014, EFSA considered the suitability of a "Qualified Presumption of Safety (QPS) approach," initially developed for the assessment of microorganisms, as a practical method for assessing botanicals and botanical preparations for which an adequate body of knowledge exists. However, in view of the high variability especially in chemical composition, only limited possibilities were seen to establish a QPS status at high taxonomic levels for botanicals. Still, the use of a developed structured assessment scheme was recommended as an extension of the 2009 EFSA guidance for the safety assessment of botanicals and botanical preparations intended to be used in food supplements (EFSA 2014).

As a further tool for risk evaluation of botanicals, EFSA listed more than 1,200 plant genus, species, and varieties in a "Compendium of botanicals reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements" (EFSA 2012b; Silano et al. 2011). Its purpose is to draw the attention of manufacturers and food safety authorities to possible safety issues when these botanicals are used in food. There is no final judgment as to whether botanicals listed in the Compendium are safe or not safe for food use.

The "Guidance for submission for food additive evaluations" (EFSA 2012c) by the EFSA Panel on Food Additives and Nutrient Sources Added to Food (ANS) is relevant for the safety evaluation of botanical preparations used as food additives. It reflects widely the principles of the "Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements" presented above. In the safety evaluation of food additives of botanical origin for use in infant formula, such as certain thickening agents (e.g., guar gum, locust bean gum, xanthan gum), an additional EFSA guidance considering the specific vulnerability of infants below 16 weeks of age has to be taken into account (EFSA 2017b).

The existing guidance for the use of botanical preparations as flavorings is dealt with in ► Chap. 91, "Risk Assessment of Food Additives."

Legal Background

The use and the occurrence of plants in food are heterogeneous and therefore subject to different national and international regulatory frameworks, which in general are based on the outcome of the scientific risk assessments of competent national or international authorities, such as the Joint FAO/WHO Expert Committee on Food Additives (JECFA) or the EFSA.

The Codex Alimentarius Commission, established by FAO and WHO in 1963, provides a global framework. It develops harmonized international food standards, guidelines, and codes of practice to protect the health of the consumers which are also relevant for botanical components in food. For food additives of botanical origin, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) publishes specifications and safety evaluations including health-based guidance values (e.g., curcumin (E 100): JECFA 2004, 2006). On the basis of these monographs, the Codex Committee on Food Additives may endorse permitted maximum levels for individual food additives. Another Codex panel, the Codex Committee on Contaminants in Foods, establishes or endorses permitted maximum levels or guideline levels for contaminants including naturally occurring toxicants in food and feed. For grains and pulses, the Codex Alimentarius standards state, e.g., that they shall be free from the following toxic or noxious seeds in amounts which may represent a hazard to human health: crotalaria (*Crotalaria* spp.), corn cockle (*Agrostemma githago* L.), castor bean (*Ricinus communis* L.), and jimson weed (*Datura* ssp.) (CODEX STAN 153-1985; CODEX STAN 171-1989; CODEX STAN 172-1989; CODEX STAN 199-1995; CODEX STAN 201-1995).

In Europe the use of botanicals and botanical preparations in food is regulated by national and EU legislations, where mainly the latter will be addressed in the following.

Overall botanicals and botanical preparations found in food fall under the basic regulations of the General Food Law (Regulation (EC) No. 178/2002/EC), in which Article 14 protects against unsafe food and which attributes responsibility for the safety of the products on the market to the food business operators.

It is known that conventional plant-derived food, e.g., certain fruits, vegetables, herbs, and spices, may contain a variety of naturally occurring substances of concern, such as α-solanine, furocoumarins, cyanogenic glycosides, capsaicin, coumarin, estragole, and thujone (e.g., Dusemund et al. 2017b). With a normal diversified diet, the intake of these substances generally only amounts to low levels not known to cause any health damage in humans. However, for preventive action, national authorities are informing the public by communicating nutritional recommendations to avoid potential risks of high or excessive individual consumption of a certain foodstuff, e.g., to consume cassia cinnamon, containing relatively high levels of the hepatotoxic coumarin only in moderate amounts (BfR 2012b), or advising against excessive intake of extremely hot chili sauces with high concentrations of capsaicin, which may cause irritation of mucous membranes, nausea, vomiting, and hypertension (BfR 2011b). Specific regulations exist for some of the abovementioned naturally occurring food ingredients, but they refer only to

flavoring purposes (Regulation (EC) No. *1334*/2008). They aim at avoiding an increase of exposure not allowing to add the isolated substances to food (e.g., capsaicin, coumarin, estragole, thujone) and restricting their concentration in compound food containing flavorings or flavoring food ingredients (coumarin, estragole, thujone). Further details on the regulations of flavorings in general are given in ▶ Chap. 91, "Risk Assessment of Food Additives."

Regarding plant-based food additives, present uncertainties regarding the differentiation between natural food colors, being food additives (e.g., beetroot red (E 162), an extract of beetroots), and coloring foods (e.g., concentrated cherry juice), considered to be normal characteristic natural food ingredients, are noteworthy. In contrast to the latter, food additive colors undergo an approval procedure (Regulation (EC) No. *1331*/2008), need labeling as an additive (Directive 2000/13/EC), and have to meet the purity criteria set out in Commission Regulation (EU) No. 231/2012. A criterion to determine whether or not a food color is an additive is that of "selective extraction of the pigments" which is per definition associated with the manufacturing of a food color considered as an additive (Annex I of Regulation (EC) No. *1333*/2008). Further details on regulations of food additives in general are outlined in ▶ Chap. 91, "Risk Assessment of Food Additives."

Regulations for novel foods and genetically modified foods of botanical origin are not presented here but in ▶ Chap. 93, "Risk Assessment of Genetically Modified Food and Feed."

With respect to food supplements, the use of botanicals and botanical preparations has not yet been harmonized within the EU but is regulated in the Member States by differing national rules. Partly, (positive) lists of safe botanicals and/or (negative) lists of botanicals which are banned or restricted for food use have been established. In some EU Member States, plant-based food supplements are subject to a notification procedure before being introduced to the market (Bast et al. 2002). The EU Directive 2002/46/EC gives general provisions for food supplements and specifically addresses modalities for use of vitamins and minerals listed in the annexes to this directive. It also allows the use of "other substances with a nutritional or physiological effect," for which no further definition or regulation is given. However, it is generally understood that this term could include botanicals and botanical extracts besides substances such as amino acids, enzymes, pre- and probiotics, and essential fatty acids (Silano et al. 2011). Furthermore, there is an announcement in the eighth recital of this directive that specific rules concerning these other substances as ingredients of food supplements should be laid down at a later stage, provided that adequate and appropriate scientific data become available.

If a harmful effect on health is suspected for a botanical or botanical preparation, a procedure based on Article 8 of the food fortification legislation (Regulation (EC) *1925*/2006) may be initiated by the European Commission which may result in a placement of the botanical (preparation) in Annex III of this regulation. On the basis of a risk assessment by EFSA, it can be banned (Annex III, Part A), restricted in use (Annex III, Part B), or in case of uncertainties reevaluated on the basis of additional safety data (Annex III, Part C). So far, following demands of EU Member States, only ephedra herb and its preparations originating from *Ephedra* species

(Annex III, Part A) and yohimbe bark and its preparations originating from yohimbe (*Pausinystalia yohimbe* (K. Schum) Pierre ex Beille) (Annex III, Part C) have been regulated by this means on the basis of existing EFSA opinions (EFSA 2013a, b).

Nutrition and health claims regarding botanical food supplements are regulated by Regulation (EC) 1924/2006. This regulation does not foresee an assessment of the safety of the product carrying the claim.

As far as botanical contaminants are concerned, Article 2 of the Council Regulation (EEC) No. 315/93 stipulates that, where necessary, maximum tolerances for specific contaminants shall be established in order to protect public health. Thus, Commission Regulation (EC) No. 1881/2006 lays down maximum levels for certain contaminants in foodstuffs, but regarding natural toxicants at present, it only includes various mycotoxins. Regarding natural toxicants, besides various mycotoxins including ergot alkaloids, only tropane alkaloids and erucic acid are addressed, EFSA risk assessments being available for both contaminants (EFSA 2013c, 2016).

In the USA the use of botanicals in food supplements (dietary supplements) is regulated under the Dietary Supplement Health and Education Act of 1994 (DSHEA), which places the burden on the Food and Drug Administration (FDA) to prove that a dietary supplement presents a significant or unreasonable risk of illness or injury under the labeled conditions of use. Under certain conditions a notification process is required for new dietary ingredients that were not marketed before October 15, 1994. The law requires the collection of all adverse event reports by manufacturers, distributors, and retailers of dietary supplements and the reporting of serious adverse event reports to the FDA. Due to case reports on adverse effects, FDA published a final rule on February 11, 2004, declaring dietary supplements that contain ephedrine alkaloids adulterated on the basis that these products present an unreasonable risk of illness or injury (FDA 2010).

Future Perspectives

Since plant-based preparations are widely present in different categories of food commodities with expanding market volume and increasing exposures of consumers, the need for a better chemical and toxicological characterization and a harmonized systematic approach of scientific risk assessment of botanicals and botanical preparations is growing.

The establishment of the EFSA guidance document for the safety assessment of botanicals and botanical preparations supplemented in 2014 (EFSA 2009b, 2014) was a first step to achieve similar standards in safety evaluations performed by the national competent authorities of Member States. The next step would be to determine systematically which botanicals should be evaluated with priority considering significant levels of substances of concern, reports on adverse effects, increases of intake rates, and negative lists of Member States (EFSA 2009b).

While the EU legislation is covering the use of botanical additives and flavorings, a general EU-wide approach is lacking for plant-based food supplements. Especially

for botanicals or botanical preparations used in products, where the borderline between food supplements (not subjected to an authorization procedure in the EU) and medicinal products (subjected to an authorization procedure in the EU) is difficult to define, continuing measures are desirable to guarantee that intakes of toxicologically relevant botanical components are within safe limits. Thus, in addition to ephedra herb and its preparations originating from *Ephedra* species (Annex III, Part A) and yohimbe bark and its preparations originating from yohimbe (*Pausinystalia yohimbe* (K. Schum) Pierre ex Beille) (Annex III, Part C), further botanicals and botanical preparations may be listed in the future in Part A, B, or C of Annex III of Regulation (EC) 1925/2006 in accordance with its Article 8. In addition activities are expected with respect to the definition of coloring foodstuffs. A catalogue of clear criteria will presumably soon be established on how to distinguish coloring foods, which are not requiring a safety evaluation and an approval, from additive food colors which need an authorization. With respect to consumer safety, unambiguous definitions of coloring foods and their uses are considered to be necessary to ensure that their application does not lead to toxicologically unacceptable higher exposures with coloring components, accompanying substances, and impurities of these sources than would result from their normal dietary intake. Legal definitions and specific provisions regarding the use of coloring foods might be necessary.

Furthermore, other EFSA risk assessments of botanical contaminants, namely, of opium alkaloids in poppy seeds and of pyrrolizidine alkaloids in food and feed (EFSA 2011b, 2017b), may result in legal consequences. A decision by the European Commission may be taken if in these cases there is a need for legal measures in the frame of Council Regulation (EEC) No. 315/93 laying down community procedures for contaminants in food to protect public health. Other measures may also be taken, such as the development of codes of practice. This has been especially recommended in a discussion paper by the Codex Committee on Contaminants in Foods to prevent and reduce pyrrolizidine alkaloid contamination of food products (FAO/WHO 2011).

Cross-References

► Risk Assessment of Food Additives
► Risk Assessment of Genetically Modified Food and Feed

Endnotes

1. This term refers to plants, including algae, fungi, and lichens, and parts of plants as a whole or cut.
2. This term refers to preparations obtained by all kind of processing, e.g., pressing, extraction, fractionation, concentration, drying, and/or fermentation.

3. This guidance was replaced in 2012 by the "Guidance for submission for food additive evaluations by the EFSA Panel on Food Additives and Nutrient Sources Added to Food (ANS)" (EFSA 2012c).

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Risk Assessment of Genetically Modified Food and Feed

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Albert Braeuning, Hermann Broll, Andreas Eisenreich, Jakob Menz, Birgit Niemann, and Bernd Schäfer

Contents

Abstract

In the European Union (EU), genetically modified (GM) food and feed are subject to strict legal requirements. Thus, food and feed products derived from genetically modified organisms (GMO) may only be placed on the market if they have previously undergone a safety assessment. According to the concept of "substantial equivalence," the safety assessment is centered on the comparison of the GM food and feed with non-GM food and feed that have a so-called history of safe use. The current approach usually comprises a molecular characterization of the genetic modification, agronomic and phenotypic analyses, as well as comparative analyses of nutritional, anti-nutritional, and toxic or allergenic constituents. Depending on identified changes in the composition, further investigations may be necessary to prove that a GMO-derived product is as safe as the non-GM comparator. Irrespective of this, feeding studies in rodents are usually required to account for the possibility of unforeseeable health effects. In the following, the general principles of the human health risk assessment as part of the market

A. Braeuning · H. Broll · A. Eisenreich · J. Menz · B. Niemann · B. Schäfer (✉)
Department "Food Safety", German Federal Institute for Risk Assessment (BfR), Berlin, Germany
e-mail: albert.braeuning@bfr.bund.de; Hermann.broll@bfr.bund.de; andreas.eisenreich@bfr.bund.de; jakob.menz@bfr.bund.de; Birgit.Niemann@bfr.bund.de; bernd.schaefer@bfr.bund.de

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authorization process by the example of food and feed derived from GM plants are illustrated, and the current legislation framework on GM food and feed in the EU is summarized.

Keywords

GMO legislation · GMO authorization · GM food · GM feed · Food and feed safety · Risk assessment · New Breeding Techniques (NBT) · Regulation (EC) No. 1829/2003 · Regulation (EU) No. 503/2013

Introduction

The first commercialized genetically modified crop was the so-called Flavr Savr™ tomato entering the US market in 1994. By introducing an additional antisense copy of the gene expressing the enzyme polygalacturonase into the genome of tomatoes, the ripening process was supposed to be slowing down preventing the softening of tomatoes and consequently the susceptibility for damage. The US Food and Drug Administration (FDA) evaluated all information related to safety submitted by the applicant, the company Calgene, and concluded that the tomato "is as safe as tomatoes bred by conventional means." This statement was based on the risk assessment principle established a bit earlier by a working group of the Organisation for Economic Co-operation and Development (OECD) dealing with safety in biotechnology (OECD 1993). The OECD concept of "substantial equivalence" takes the idea that existing food products with a so-called history of safe use can serve as the starting point for comparison of the safety assessment and the nutritional values of a food product that has been modified using biotechnological methods. Consequently, in case a genetically modified food product is found to be substantially equivalent to an existing product, no safety concerns other than for the existing product are expected. On the contrary, in case the new product is considered not to be substantially equivalent to its appropriate non-genetically modified (GM) comparator, it does not necessarily indicate that it is unsafe. It just indicates to the assessors different characteristics that need to be evaluated in a more targeted manner to conclude on the safety of the respective product (OECD 1993).

Globally, in at least 26 countries, more than 190 million hectares of genetically modified organisms (GMO), mainly soybeans, maize, cotton, and rapeseed, were cultivated in 2018. Beside these big "four" also potatoes, papaya, sugar beets, squash, and eggplant are approved and on the market in various countries. In terms of GM food and feed, more than 70 countries do have legislation in place regulating risk assessment and authorization of products derived from GMO (ISAAA 2018).

Until now, there are no commercially available genetically modified farm animals approved and commercially grown around the world with the exception of the AquAdvantage Atlantic salmon bearing a growth hormone-regulating gene from

the Pacific Chinook salmon, together with a promoter from the ocean pout in its genome. Although approval for human consumption has been granted by the Canadian and US authorities already in 2015, it still not reached US consumers due to court cases not finally solved yet. However, many new genetically modified animal traits are under development and foreseen for commercialization. Since the use of GMO animals is still considered to be marginal in comparison to the vast amounts of biotech crops approved and cultivated around the world, this chapter is mainly focusing on GM plants.

Legislation Framing the Use of GMO in the European Union

According to European legislation the term "genetically modified organism (GMO)" means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. However this includes organisms obtained through certain techniques of genetic modification such as mutagenesis and cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through "traditional breeding methods" (European Union 2003). In practice, it means that genetic elements from different sources are combined and introduced into the genome of a host organism resulting in a specific GM event since the location of the event in the genome of the GMO could not be predicted before the "New Breeding Techniques (NBT)" have been developed.

Since 1990 GMO are regulated for the deliberate release into the environment in the European Union (EU) (European Communities 1990a). In parallel also legislation came into force regarding the contained use of genetically modified microorganisms (European Communities 1990b). The latter one is out of the scope of this book chapter; nonetheless, it is worth mentioning that a comprehensive legislation has been established considering the whole cascade starting from research on GMO in the laboratory up to the commercialization of GMO on the EU market.

Nowadays, the deliberate release of GMO into the environment is regulated by Directive 2001/18/EC and includes, besides the risk assessment described in detail below, also a comprehensive environmental impact assessment (European Union 2001). Since GMO which are deliberately released into the environment are potentially unable to get back into a contained use, particular attention to the environmental impact is needed. The main questions to be answered are the potential for surviving in the environment and the possibility to cross with native species in the EU. In the EU, only a single GMO, an insect-resistant GM maize line called 'MON810,' is currently approved for cultivation. However, its cultivation for commercial purposes is currently only done in the north of Spain.

In 2015, after long debate about reservation against GMO cultivation in the public in different EU member states, the new Directive (EU) 2015/412 has been adopted allowing EU countries to restrict or prohibit GMO cultivation on their territory by "invoking compelling grounds such as environmental or agricultural policy objectives, town and country-planning, land use, coexistence, socio-economic impacts, or

public policy." Seventeen member states and two autonomous regions took the opportunity to declare their territory or parts of their territory to be excluded from commercial cultivation of GM plants. However, it is not clear yet, if the goal to motivate member states granting the authorization of new GMO will be achieved. In the future, it remains to be seen if new European Commission (EC) proposals based on European Food Safety Authority (EFSA) favorable risk assessment opinions for the authorization of GMO will be positively voted by member states in the respective expert committee (Eriksson et al. 2019).

In 2003, with the adoption of Regulation (EC) No 1829/2003, specific legislation on genetically modified food and feed was implemented covering risk assessment and labeling of products thereof (European Union 2003). Further specifications have been introduced with Regulation (EU) No 503/2013 providing detailed information on the molecular characterization; the agronomic/phenotypic and compositional analyses; the toxicological, allergenic and nutritional characterization; and finally the environmental consideration of the genetically modified food or feed (European Commission 2013). Applicants need to follow these requirements and submit a complete dossier to the centralized risk assessment process led by the EFSA located in Parma, Italy (Fig. 1). According to the EU law, a cross between two authorized GMO is resulting in a new so-called stacked GM event that needs a separate application including a new risk assessment. Consequently, the whole EU legal framework regarding GMO is considered to be process-based in contrast to a product-based approach as it is implemented by countries outside the EU such as Argentina, Canada, and the USA.

As a prerequisite before entering the EU market, "genetically modified food and feed must not (a) have adverse effects on human and animal health or the

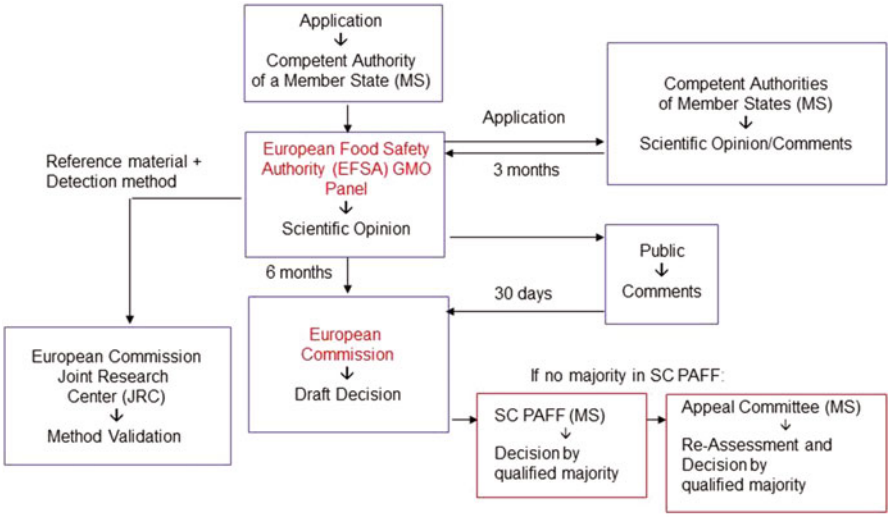

Fig. 1 Authorization procedure for GM food and feed. The final decision upon the authorization is made by the Standing Committee on Plants, Animals, Food and Feed (SC PAFF)

environment; (b) mislead the consumer; (c) differ from the food which it is intended to replace to such an extent that its normal consumption would be nutritionally disadvantageous for the consumer" (European Union 2003).

Consequently, applicants need to provide all studies necessary to support the safety of a GM food and feed intended for marketing in the EU. Such dossier can be submitted to a competent authority of any EU member state which is subsequently passing on the application to the EC for its consideration. The EC is requesting EFSA to carry out the risk assessment within a time frame of six months. However, the time needed to finalize the risk assessment is often much longer, on average about two years. This is due to the fact that for almost all applications, the EFSA is requesting clarifications on the dossier submitted or is requesting the applicant to provide new or additional studies needed to carry out a proper risk assessment. While the applicant is preparing the answers to EFSA questions, the clock for the application is stopped and will be restarted when EFSA receives the answer from the applicant. Using the EFSA opinion as a basis, the EC is preparing a draft proposal either for granting an authorization in case of a positive opinion or refusing the authorization in case the EFSA opinion is negative. The final decision is made in a member states expert committee comprising representatives from all member states. If no concluding decision is agreed upon (adopted or not), the EC is asking the so-called Appeal Committee, which also consists of representatives from all member states, to discuss again the draft decision. In most cases, member states were not able to vote either for or against the adoption of an EC draft decision. In these cases, the EC is adopting the draft decision, and the GM food and feed will have the right to enter the EU market. Up to the end of 2019, food and feed derived from 73 different GM plants are approved for the EU market. Those GM food and feed have to be labelled as "genetically modified" with the purpose to inform consumers about the respective ingredient. Since the implementation of legislation regarding GMO, new and more efficient methodologies for the specific modification of genomes have been developed. These methodologies are summarized under the term *New Breeding Techniques* (NBT). The most prominent example is called *Clustered regularly interspaced short palindromic repeats* (CRISPR) and CRISPR-associated (Cas) systems. Besides the introduction of foreign deoxyribonucleic acid (DNA) similar to what can be achieved by classic techniques of genetic modification, using different NBTs, it is possible to introduce a site-directed mutation resulting in an organism without foreign DNA but with a modification in an individual gene or in a regulatory DNA sequence. Many examples of such modified plants and animal species are described in the scientific literature.

In 2014, the European Court of Justice (ECJ) was asked by the French Council of State, if mutagenesis as it can be achieved using NBT leads to GMO and if such organisms fall under the regulations laid down by Directive 18/2001/EC. In July 2018, the ECJ came to the conclusion that organisms obtained by mutagenesis using techniques that alter the genetic material in a way that does not occur naturally are considered to be GMO. The only exemptions from this rule are "organisms obtained by means of techniques/methods of mutagenesis which have conventionally been used in a number of applications and have a long safety record" (ECJ 2018). The

ECJ further specifies in its argumentations that those techniques developed and applied before the respective Directive 2001/18/EC came into force are the application of chemical or physical mutagenic agents that have a long history of safe use (ECJ 2018). For further details on this issue, please refer to Broll et al. (2019). It needs to be mentioned that from a scientific point of view, it is impossible to distinguish whether a single mutation is derived from the application of a NBT, was conventionally introduced using chemical or physical mutagenic agents, or results from a spontaneous mutation as it is happens naturally in genomes as part of evolution. However, the regulation requires the labeling of GM food and feed after it is authorized in order to provide to the consumer an informed choice (European Union 2003). Therefore, it is necessary to provide along with the application an unambiguous identification possibility to control correct labeling. "Classical" GMO containing new foreign DNA fragments can be detected applying routine diagnostic tools such as polymerase chain reaction (PCR) established in many laboratories around the EU.

Assessment Procedure for Food and Feed from GM Plants

The safety assessment of a food product relative to an appropriate non-GM comparator considers at least three aspects: (i) intended and unintended effects; (ii) the identification of new hazards; and (iii) the identification of changes, which are relevant to human health including key nutrients. While the term "intended effects" covers basically the genetic modification introduced into the plant genome and the intended modifications in plant biochemistry resulting from that modification, unintended effects might happen due to genetic rearrangements during the process of genetic modification and subsequently result in metabolic perturbations.

As mentioned above, "substantial equivalence" is basically describing the characteristics of a GM plant compared to a conventional product. However, nowadays, it is considered more appropriate to use "comparative analysis" or "comparative approach" because these terms refer to the whole risk assessment process rather than focusing explicitly on the final outcome. The risk analysis comprises the agronomic and phenotypic characteristics, composition, toxicity, allergenicity, and nutritional values of the GM plant in comparison to a non-GM comparator genetically as close as possible. An overview over the assessment procedure for GM food and feed is given in Fig. 2.

Hazard Identification and Characterization

Molecular Characterization

An initial molecular characterization of all genetic modification events in GM plants is indispensable, regarding the identification of intentionally as well as unintentionally generated biologically relevant changes, especially those that might raise safety concerns. This includes – among other things – information on

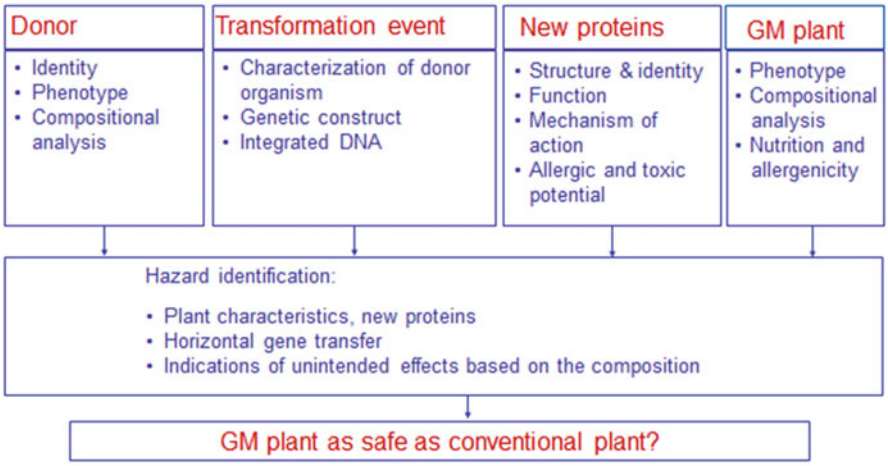

Fig. 2 Assessment procedure for GM food and feed

the introduced gene, the donor, data on consumption history of the newly expressed protein(s) by humans and animals, as well as information on potential similarities with proteins, which may induce/mediate adverse effects.

Consequently, for the assessment of the molecular characteristics of the GM plant in question, the nature and source of the genetic modification needs to be analyzed. In particular the organisms (donors) from which the genetic elements derived need to be characterized regarding their allergenic potential and toxicity. Therefore the applicant is requested to run specific bioinformatic analyses of the DNA fragments/genes and new proteins transferred to the genetically modified organism using databases including DNA sequences of potential allergens and toxins.

Using up-to-date databases, the resulting amino acid (AA) sequence, obtained from the DNA sequence of the insert and the regions spanning its junction sites, have to be analyzed regarding homologies to sequences of known allergens and toxins via adequate in silico methods (bioinformatics).

Besides characterization of the toxicological and potential allergenic potential, it is also important to analyze the potential for horizontal gene transfer from the GM plant either to humans, animals, or microorganisms. In cases intact DNA fragments are transferred horizontally, the risk remains that organism uptaking the foreign DNA, e.g., a gene responsible for an herbicide tolerance.

For the molecular characterization, a detailed description of the development of the GM plant needs to be provided by the applicant. This includes the description of the vector(s) used for the transformation process with all the individual elements and their function(s) ("mode of action") in the organism(s) they are derived from and how they have been introduced into the genetic construct. The applicants are requested also to provide the whole DNA sequence of the actual insert into the host genome including the sequence of the integration site. Bioinformatic analyses have to be carried out at the DNA as well as AA sequence level. DNA-based in silico

approaches are used to identify possible genetic rearrangement of the inserted construct or at the genomic site of insertion. Furthermore, the DNA sequence is used to check for the absence of disruption of endogenous genes, as well as for the presence of putative novel open reading frames, which might have been formed as a consequence of the insertion of the foreign genetic material.

AA sequences from the GM plant are compared to sequences already described as potentially allergenic or toxic in well-known up-to-date databases. Relevant similarities will need to further detailed investigations, which might also include additional laboratory work or animal feeding studies in case a valid hypothesis for a concern can be formulated. In case the new GM plant expresses foreign proteins due to the genetic modification, analytical investigation of the respective proteins in the plant material is mandatory. Determination of certain ribonucleic acids and/or intermediary metabolites may be relevant if the genetic modification is intended to lead to the silencing of a certain gene or if the modulation of metabolic pathways in the GM plant is intended. Information regarding the stability of the new traits over at least five generations is also needed.

In case single events were crossed to obtain so-called stacked events, the risk assessment is focused on the potential interaction between the individual genes or gene products of the respective single events to identify any synergistic, antagonistic, or additive effect. Therefore, the structure of the inserts in the stacked event is compared to the structure in the single events. Moreover, comparative data on gene expression are required and will provide important information if any newly inserted gene is up- or downregulated due to potential interactions.

Comparative Analyses

Thorough comparative analyses of the composition of genetically modified plants are conducted, as well as of their phenotypic and agronomic characteristics, which aim to identify differences between the GM plant and derived food and feed and its most appropriate non-GM comparator. In the best case, the parental line used for the genetic modification can be used as the non-GM comparator. For the comparative analysis, the application of two separate tests is needed: The test of difference is used to clarify, if the GM plant, besides the intended modification, is statistically significant different from its non-GM comparator. The test of equivalence is used to identify if the properties of the GM plant are considered within the range of natural variation, as is determined based on a set on non-GM reference varieties which do have a so-called history of safe use. For herbicide-tolerant GM plants, it is necessary to additionally examine whether treatment with the intended herbicide(s) affects the plant's characteristics.

Applicants should conduct at least eight field trials within the receiving environment in which the GM plants will be grown for commercial purposes. At each site, the GM plant either treated with the intended herbicide or with conventional herbicides only will be planted along with the non-GM comparator and additional non-GM plants suitable for the respective regions.

Regulation (EU) no 503/2013 provides further detailed guidance on the selection of comparators, field trials design, and the appropriate statistical methods in the

comparative analyses. Those parameters that are identified to show statistically significant differences between the GM plant and its non-GM comparator and lack of equivalence with non-GM plants taking into account natural variation need to be analyzed further in the safety assessment (see sections on "Toxicological Assessment," "Assessment of Allergenicity," and "Nutritional Assessment").

For the identification of variations in the phenotype and agronomic characteristics, a comparison of parameters including yield, plant morphology, flowering time, plant height, growing degree days to maturity, kernel weight (maize or rape seed), duration of pollen viability, response to plant pathogens and insect pests, and sensitivity to abiotic stress are compared between the GM plant and its conventional counterpart taking into account also the natural variation determined by the additional non-GM plants growing in the same filed trial. Regarding the analysis of the composition, an 'OECD Working Group for the Safety of Novel Foods and Feeds' has been established and is continuously elaborating so-called Consensus Documents on compositional considerations for new varieties of crops identifying key components that might be used for the analysis. Ranges of variation are also provided. The parameters comprise key components such as micronutrients (vitamins and minerals), macronutrients (fat, protein, carbohydrates), natural toxins, antinutrients, as well as allergens and other plant metabolites typical for the plant species. For oil-rich plants, a fatty acid profile needs to be determined, as well as an AA profile for plants used as an important source of protein. In case the introduced trait results in a modification of a metabolic pathway, the analysis of metabolites of the metabolic pathway(s) affected has to be carried out. The comparative analyses are only able to detect differences in known characteristics or parameters as described above. New non-targeted profiling techniques (the so-called "Omics" techniques such as genomics, transcriptomics, proteomics, and metabolomics) need to be further examined before their use in comparative analyses (EFSA 2011a). In particular, the reliability and comparability as part of a thorough validation process need additional efforts in order to generate results that can be reproduced independently.

Toxicological Assessment

Targeted genetic manipulation of plants leads to changes in the composition of generated GM plants and consequently also of derived food and feed. Since this could potentially affect human and animal health, the toxicological impact of the genetic modification has to be evaluated. In this context, it is mandatory to consider all – intentionally as well as unintentionally – generated biologically relevant changes in GM plants. This includes the presence of new constituents, such as proteins, metabolic products or regulatory ribonucleic acids, as well as alterations of endogenous constituent levels beyond normal variation.

Newly Expressed Proteins

Proteins are commonly present in most food and feed. They are of high nutritional relevance and only few of them induce adverse effects after oral uptake. Nevertheless, for GM plants and derived food and feed, an evaluation of the potential toxicity

and allergenicity of newly expressed proteins has to be done (European Commission 2013). In this context, the AA sequence, molecular weight, post-translational modifications (e.g., glycosylation), and biological function of the protein should be characterized. If the protein is enzymatically active, the substrate specificity and possible reaction products should also be considered. To investigate possible sequence similarities with known toxins or allergens, bioinformatic analyses using up-to-date databases should be performed. Depending on the results, further analyses may be needed to complete the risk assessment. Additionally, the evaluation should consider protein stability a) under conditions relevant for processing and storage of food and feed derived from the GM plant and b) against digestive enzymes, such as pepsin as well as potential interactions between the newly expressed proteins and other plant constituents (European Commission 2013).

Whether further toxicological studies are needed or not is determined by a) available information on a history of safe consumption and b) the results of the aforementioned evaluation. No additional toxicological analyses are necessary if a proper use and safe consumption as food and feed of the plant as well as of the newly expressed proteins is duly documented. However, if the available toxicological information is insufficient or raises safety concerns, specific toxicity testing of newly expressed proteins is needed (European Commission 2013). In this context, a repeated dose 28-day oral toxicity study using rodents should be conducted according to the OECD Guideline 407 (OECD 2008). Depending on the results of that study, further targeted analyses may be required. Moreover, if there is a possibility for synergistic or antagonistic interactions between two or more newly expressed proteins that may affect safety, additional studies with combined administration of these proteins should be performed. Due to the limited availability of plant-produced test material, the specific toxicity of newly expressed proteins is often assessed using a substitute material generated via microbial expression systems. In this context, it is necessary to provide adequate evidence for structural and functional equivalence of the microbial substitute compared the protein expressed in GM plants, including the AA sequence, molecular weight, post-translational modifications, immunological reactivity, and, if applicable, the enzymatic activity.

New Constituents Other than Proteins

As mentioned before, genetic modifications, such as insertion of a specific DNA sequence in GM plants, can lead to the generation of new constituents other than proteins, such as metabolic products or regulatory ribonucleic acids. In this context, the generation of GM plants that are resistant to specific pathogens due to the synthesis of specific secondary metabolites is also an important scope. For all identified new constituents, a safety assessment has to be done, including an evaluation of their toxic potency, which may also require toxicological analyses. The specific testing strategy should be selected according to the EFSA guidance for food additive evaluations (EFSA 2012a), as described in ► Chap. 91, "Risk Assessment of Food Additives" Toxicological testing is not necessary if there is a documented history of safe use and consumption as food and feed of the respective constituent.

Altered Levels of Endogenous Constituents

Genetic modification may affect levels of endogenous constituents beyond normal variation, such as modification of fatty acid patterns in seeds of maize or rape used for vegetable oil production. However, besides intended modifications, unintentionally generated changes of biological relevance may be associated with the genetic modification, which also need further evaluation. The safety of any altered content of food and feed constituents, such as macro- and micronutrients, natural toxins, or other plant metabolites, has to be adequately evaluated. Therefore a risk assessment is required, based the knowledge of the physiological function, the potential toxicity, and the anticipated changes in intake levels of the respective constituent. Depending on the results, further toxicological testing may be required to complete the risk assessment.

Assessment of Whole Food and/or Feed Derived from GM Plants

According to Regulation (EU) No 503/2013, testing of the whole food and/or feed derived from GM plants is mandatory. This is also the case if the preceding molecular, compositional, or phenotypic evaluations lead to indications for potential occurrence of unintended effects. For GM plants containing stacked events, an assessment of whole food and/or feed is also required if the abovementioned evaluations indicate possible interactions between the events stacked within the plant. The assessment of whole food and/or feed usually includes a modified subchronic 90-day feeding study in rodents based on the principles of OECD guideline 408 (EFSA 2008; OECD 2018). This type of study is considered to offer sufficient specificity and sensitivity to characterize toxicologically relevant differences as well as nutritional deficiencies which may be induced by genetic modification, including intentionally as well as unintentionally generated effects (EFSA 2008). In 2011, EFSA published a document providing guidance regarding design, performance, and evaluation of such studies (EFSA 2011b). Among other things, this guidance document provides advice regarding the preparation of appropriate test diets, dose selection, animal housing and determination of sample size, statistical analysis, as well as the interpretation of study data. Depending on the results of the subchronic study, additional toxicological studies may be required, such as studies on reproductive effects or chronic toxicity. Further information regarding the possible occurrence of unintended effects may be obtained from additional studies with young rapidly growing animal species, such as broiler chickens as an animal model for non-ruminants (EFSA 2011a).

Over the past decade, the necessity and design of feeding trials in the context of GMO risk assessment has been subject of a lively discussion. The debate was further stimulated by the publication of a controversial study on potential chronic effects of a herbicide-tolerant GM maize, which was retracted after its initial publication and subsequently republished elsewhere (Séralini et al. 2014). In this context, the scientific value of animal feeding trials and the potential health risks of GM maize were intensively studied in three research projects (GRACE, G-TwYST, GMO90+) (Zeljenková et al. 2014, 2016; Coumoul et al. 2019; Steinberg et al. 2019). The general conclusions of these activities were that a) neither subchronic (90 days) nor

long-term feeding trials (0.5–2 years) revealed any health risks of the GM maize tested and b) the additional scientific value of feeding trials is very limited in the absence of a targeted hypothesis. However, after reviewing the respective information requirements in relation to the findings of the GRACE project and other scientific knowledge available by that time, as foreseen by Commission Implementing Regulation (EU) No 503/2013, the European Commission concluded to maintain the requirement for the mandatory submission of a 90-day feeding study (European Commission 2017).

Assessment of Allergenicity

Food allergies, mainly associated with immunoglobulin E (IgE)-mediated processes, are often induced by proteins contained in these foods. Therefore, the allergic potential of all proteins newly expressed in GM plants has to be evaluated applying the weight-of-evidence approach (EFSA 2010a; Codex Alimentarius 2009). In order to minimize uncertainty regarding the allergenicity assessment for those newly expressed proteins, different aspects have to be considered to gain a cumulative body of evidence for its allergic potential.

First of all, it should be analyzed whether the source of the introduced protein-coding gene exhibits an allergenic potential. In order to identify potential IgE cross-reactivity, analyses regarding structural similarities and/or homologies of the AA sequence of the newly expressed protein(s) compared to known allergens have to be done. If there is a sequence identity of $\geq$35% in a frame of at least 80 AA, further examinations are mandatory. In this regard, additional in vitro tests determining the binding capacity of the protein of interest to specific IgEs present in serum of allergic patients (specific serum screening) are required, if structural and/or sequence homologies were indicated. The same applies in the case that the source of the introduced protein-encoding gene is considered allergenic (even if the is no sequence homology to known allergens). In this context, individual sera from well-characterized allergic subjects as well as adequate immunochemical methods (e.g., enzyme-linked immunosorbent assay (ELISA) or electrophoresis followed by immunoblotting) should be utilized.

Besides the abovementioned processes, potential triggering effects of the newly expressed protein(s) on non-IgE-mediated gluten-sensitive enteropathy (also called celiac disease – CD) should be evaluated if the protein-encoding gene was derived from cereal crops, such as wheat, rye, oat, or barley. However, other non-IgE-mediated enteropathies cannot be considered yet because their pathological mechanisms are insufficiently understood (EFSA 2017). In principle, the assessment of the ability of newly expressed proteins to trigger CD follows the same strategy as the allergenicity assessment. It starts with searching of the available information regarding the source of the protein and on human exposure to the protein. If the available knowledge on the protein is insufficient to support its safety, additional considerations and in silico approaches should be applied (e.g., sequence comparisons with known CD peptides and motif searches). Furthermore, additional in vitro tests can be performed (e.g., human leukocyte antigen locus DQ (HLA-DQ)- peptide binding assays or testing with T-cell clones derived from CD patients).

Since stability to enzymatic proteolytic digestion is regarded as a characteristic of allergenic proteins, the pepsin resistance test carried out under standardized conditions is still considered as relevant additional information (even though there is no absolute correlation). However, a refined in vitro digestion test is yet under experimental validation and will replace the simple pepsin resistance test in the near future. This elaborated test includes additional conditions more representative of the gastric environment (e.g., regarding the pepsin level and pH), together with an intestinal digestion phase (EFSA 2017). Cell-based in vitro assays as well as in vivo tests utilizing animal models may also provide further relevant data. However, those tests have not yet been validated for use in the assessment of allergenicity.

Another possible unintended effect of a genetic modification may arise from insertion of the protein-coding gene in recipients, which are known to be allergenic, such as soybeans (*Glycine max*). It cannot be excluded that this could lead to elevated levels of naturally occurring endogenous allergens, consequently increasing the allergenicity of the whole food. Therefore, it is mandatory to compare the levels of relevant naturally occurring endogenous allergens of the GM plant with those of the non-GM comparator. In this context, proteomics or immunochemical analyses (e.g., ELISA utilizing sera from allergic subjects) are considered as appropriate analytical methods. According to Regulation (EU) 503/2013, the levels of allergens should be analyzed in the same way as any other complex compounds in the comparative assessment procedure. If the level of a specific allergen in a GM plant is significantly higher than the corresponding level in the appropriate non-GM comparator(s) and is not within the estimated range of natural variation, the biological relevance in relation to human health should be assessed in the light of its anticipated intake in the European population (EFSA 2017).

In order to confirm the absence of an elevated allergenic risk after market launch, post-market monitoring (PMM) may be required on a case-by-case basis. However, so far, no specific concern was formulated by EFSA in any scientific opinions on GM plants (EFSA GMO Opinions up to March 2020) requiring a follow-up by PMM.

Nutritional Assessment

Different aspects regarding the composition of the food have to be considered in the nutritional assessment. This includes the levels of nutrients and antinutrients as well as the bioavailability and biological efficacy of nutrients. Additionally, the anticipated dietary intake of the food as well as resulting nutritional impact has to be taken into account. If the comparative compositional analysis (see section on "Comparative Analyses") revealed no biologically relevant differences between the GM plant and the non-GM comparator, except for the introduced trait(s), the exposure assessment focuses on the newly expressed proteins and no further studies regarding nutritional equivalence are needed. In this case the dietary exposure estimations should cover average and high consumers across all the different age classes and special population groups and identify and consider particular consumer groups with expected higher exposure (EFSA 2019). Consumption data and guidance for the exposure assessment are provided by EFSA with the Comprehensive European Food

Consumption database covering the national food consumption surveys from 21 EU member states (EFSA 2015).

If compositional characteristics are different, an evaluation of their nutritional biological relevance is required. Sometimes, it may be sufficient to base the nutritional assessment on an estimation of the expected intake level changes regarding the relevant nutrient(s), which may result from replacement of the respective traditional food product. This can be done taking into account current dietary recommendations and nutritional reference values. In other cases, specifically designed animal studies may be needed to evaluate the biological relevance, e.g., when altered bioavailability raises nutritional concerns. The first GM plant with an intended change in nutrient levels assessed by EFSA was a soybean variety exhibiting a modified fatty acid profile in seeds. An increased content of oleic acid (C18:1) as well as reduced levels of linoleic acid (C18:2), α-linolenic acid (C18:3), and palmitic acid (C16:0) were the main differences compared to commercial non-GM varieties. The nutritional assessment for this GM plant compared to non-GM varieties focused on soybean oil, the main product for human consumption, and was based on consumption data from the UK. In this context, differences regarding the intake of relevant fatty acids were determined considering different scenarios. Among others, this included a scenario for total replacement of conventional soybean oil by oil derived from the GM variety, which reflects a theoretical extreme case, possibly leading to an overestimation of the actual intake. As anticipated, the replacement would lead to an increased oleic acid intake and to a decreased intake of palmitic acid, which is in line with current dietary recommendations (EFSA 2010b). Moreover, no nutritional concerns were identified regarding the expected decreased intake of α-linolenic acid and linoleic acid (EFSA 2012b).

Exposure Assessment

In addition to hazard-related information, the risk assessment according to Regulation (EU) No 503/2013 requires a thorough assessment of the anticipated extent of use and intake of the genetically modified food and feed. As already detailed in the context of the nutritional assessment, the exposure assessment should be based on the expected use pattern of the GM plant, and the expected intake should be estimated from representative consumption data for products obtained from the respective conventional crops (European Commission 2013). Moreover, the expected intake of new constituents (e.g., proteins) and endogenous constituents with altered levels of the GM plant should be estimated, taking into account the influences of processing, storage, and expected treatment of the food and feed (European Commission 2013).

Risk Characterization

Regulation (EU) No 503/2013 requires a comprehensive risk characterization which considers all available information from hazard identification, hazard characterization, and exposure assessment. Among other things, the risk characterization aims to

ensure that (a) the GM food has no adverse effects on human health, (b) replacement of conventional food by GM food is not nutritionally disadvantageous for the consumer, (c) the GM food does not mislead the consumer, and (d) the GM feed does not indirectly harm or mislead the consumer by altering the animal products (European Commission 2013). In this context, the probability and severity of adverse health effects and the magnitude of uncertainties in the risk assessment should also be taken into account.

Cross-References

- ▶ Microbiome Product Toxicology: Regulatory View on Translational Challenges
- ▶ Novel and Traditional Foods: Novel Food Regulation in the EU
- ▶ Risk Assessment of Food Additives
- ▶ Risk Assessment of Food Components with Botanical Origin
- ▶ Toxicological Aspects in the Regulation of Gene Therapy Medicinal Products

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Novel and Traditional Foods: Novel Food Regulation in the EU

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Julika Lietzow, Claudia Luckert, and Bernd Schäfer

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Abstract

The ongoing globalization, together with a continuing development of new technologies, has led to the emergence of novel foods seeking to enter the markets. These include exotic food from distant countries, biotechnologically gained products, edible microorganisms, or components containing new types of molecules or nanosized food components.

Safety regulations have been developed by many countries with the major aim to allow only safe novel food on the market.

In order to clearly exclude any known risks that may be associated with various nutrients and their ingredients or residues, and to avoid unknown risks that may potentially accompany novel technologies, the regulations became increasingly differentiated and complex.

J. Lietzow · C. Luckert · B. Schäfer (✉)
Department "Food Safety", German Federal Institute for Risk Assessment (BfR), Berlin, Germany
e-mail: julika.lietzow@bfr.bund.de; claudia.luckert@bfr.bund.de; bernd.schaefer@bfr.bund.de

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The present contribution briefly introduces regulations in different jurisdictions around the world, and analyzes the new European Novel Food Regulation (EU) 2015/2283 in greater detail.

It elucidates the historic evolution of the European regulation by comparing with the previous Regulation (EC) No 258/97 and presents central new aspects aiming of the new regulation to enhance the efficiency of the authorization process and reduce unnecessary trade barriers, while ensuring food safety. Along with the authorization process, the chapter elaborates the risk assessment process and, amongst others, the requirements concerning the assessment of compositional, toxicological, and allergological safety. Beyond this, the simplified notification procedure for traditional foods from third countries with a safe history of use is presented, including the respective requirements for the safety assessment.

Keywords

Novel Food Regulation · Regulation (EU) 2015/2283 · Authorization procedure for Novel Foods · Novel Food categories · Traditional foods from third countries · Safe history of use · Risk assessment · Exposure assessment · EFSA

Introduction

In general, food law does not impose any authorization or licensing requirements for the production, import, or marketing of food which is commonly consumed by the resident population. With respect to particular food, which are "new foods" on the market, great differences between the regulatory systems across the countries worldwide exist.

For example, "new foods" on the market in the **USA** are not specifically defined and do not require a premarket approval by governmental authorities. Basically, food business operators who want to bring "new foods" on the US market have a legal obligation to ensure that the foods they offer to consumers are safe under the conditions of use and in compliance with applicable legal requirements. In this so-called GRAS (generally recognized as safe) self-affirmation, the food business operator has the ability to self-affirm the safety of the Novel Food. If a food or ingredient is not very customary, its GRAS status can be affirmed by an independent panel of recognized experts. The pivotal studies used in the risk assessment must be generally available, e.g., through publication in the scientific literature. If the expert panel concludes the product is GRAS, the manufacturer may or may not consult the US Food and Drug Administration (FDA). Besides GRAS self-affirmation, the food business operator can voluntary notify the FDA of a conclusion that a substance is GRAS under the conditions of its intended use. In contrast, if a substance is used as food additive it needs approval by the FDA before retailing (details on FDA webpage (FDA (U.S. Food and Drug Administration) 2020)).

Novel foods in **Japan** are likewise not specifically defined. No premarket assessment and authorization is required for "new" food ingredients that are used as food, but compliance with the Food Sanitation Act has to be warranted. If the "new" food ingredient is a "new" food additive, a premarket assessment and authorization by the Ministry of Health, Labour, and Welfare (MHLW) is required (details on MHLW webpage (MHLW (Ministry of Health, Labour and Welfare) 2020)).

Comparable to the European Union, novel foods and novel food ingredients in **Australia and New Zealand** are specifically defined and regulated under the Food Standards Code (Standard 1.5.1). Novel foods are nontraditional foods that require premarket safety assessment by the statutory authority Food Standards Australia New Zealand (FSANZ) in order to determine their safety before they are offered to consumers. If the Novel Food passes this assessment, it is listed in the Standard and can be sold as food or used as food ingredient in Australia and New Zealand, as long as it complies with any specified conditions (details on FSANZ webpage (FSANZ (Food Standards Australia New Zealand) 2020)).

Novel foods in **Canada** are regulated under Division 28 of the Food and Drug Regulations (FDR) setting out the definition of a novel food, including foods derived from genetically modified organisms (GMOs) as well as premarket notification requirements. In the approval process the food business operator who wants to sell or advertise a novel food has to notify Health Canada, in particular the Food Directorate, and submit information regarding the product in question so that an assessment can be made by Health Canada with respect to the product's safety prior to sale (details on webpage of Government of Canada (2020)).

In the **European Union**, it is basically in the responsibility of the food business operator to ensure that food is safe when placed on the market and the requirements of food law based on Regulation (EC) No 178/2002 (2002) are met. With respect to particular food, for instance food additives, foods from genetically modified organisms (GMO), or new types of food, food ingredients or ways of producing food, specific regulations arose from the circumstances implementing premarket approval systems in the European Union. Contrary to food defined by Regulation (EC) No 178/2002, these particular foods are not considered as a priori safe. Therefore, authorization accompanied by assessment of the safety risk arising from consumption of those foods is regarded as mandatory to achive a high level of protection of human health and of consumers.

Hence, the regulation of novel foods and novel food ingredients evolved among others in response to applications of new technologies in the food sector (e.g., use of genetic modification in food) or exotic foods which may be traditional with a long history of use outside the EU as well as the continuous search for new sources of nutrients. On 15 May 1997, Regulation (EC) No 258/97 (1997), known as Novel Food Regulation, came into force to harmonize national procedures and to introduce a statutory approval system for Novel Foods across the European Union. The stated objective of the regulation was to ensure food safety and protection of human health related to new products or ingredients

intended for human consumption on the European market. To enable integration of recent developments in Union law and technological progress and to simplify the current authorization procedures, the Union's rules on Novel Foods initially established by Regulation (EC) No 258/97 of the European Parliament and of the Council and by Commission Regulation (EC) No 1852/2001 (2001) were replaced by the new Novel Food Regulation (EU) 2015/2283 (2015).

Regulation of Novel Foods in the European Union

Previous History: Situation Before 1 January 2018

Before 1 January 2018, novel foods were recently governed by the Regulation (EC) No 258/97 establishing a mandatory, decentralized premarket authorization system. The definition of Novel Food was based on two cumulative criteria that had to be fulfilled. The first defining criterion was that the food or ingredient was not used for human consumption to a significant degree within the European Union before the introduction of the legislation on 15 May 1997. The second defining criterion implied that the food had to fall within one of the defined food categories explicitly mentioned in Article 1 of Regulation (EC) No 258/97 (latest consolidated version from 07.08.2009).

Prior to placing a food or food ingredient falling under this regulation on the Community market an application in accordance with Commission Recommendation 97/618/EC (1997) concerning the scientific information and the safety assessment report was required. The risk assessment process involved an initial assessment by a Member State and if no objections were raised and no additional assessment was required, the novel food could be placed onto the market. In case of reasoned objections by another Member State or the European Commission to the initial assessment, the EC's Scientific Committee on Food (SCF)[1] or the European Food Safety Authority (EFSA) was consulted.

Based on the positive or negative opinion of this expert panel, an authorization decision had to be taken by the European Commission assisted by the Standing Committee on the Food Chain and Animal Health (SCFCAH) (previously known as the Standing Committee for Foodstuffs) consisting of Member States' representatives. According to experience, duration of the authorization process could take several years (e.g., decision on Chia seeds – 6 years (Commission Decision 2009/827/EC 2009); decision on Baobab dried fruit pulp – 2 years (Commission Decision 2008/575/EC 2008); and Decision on synthetic Zeaxanthin – 9 years (Commission Implementing Decision 2013/49/EU 2013)).

If novel foods or novel food ingredients were considered to be "substantially equivalent" to an existing food or food ingredient already available on the European market with regard to their composition, nutritional value, metabolism, intended use, and the level of undesirable substances, only a notification to the European Commission was required.

The New Novel Food Regulation (EU) 2015/2283: What Is Actually New?

Following Article 14 of the Regulation (EC) No 258/97, a revision was initiated after evaluation of the regulation which discussed issues that had emerged due to the practical implementation. Stakeholder consultations on a European Commission discussion document and a subsequent evaluation emphasized the need for an update and revision of the former provisions for Novel Food (EC (European Commission) 2008). To take into account the scientific and technological developments since 1997, it was necessary to specify and update the existing Novel Food categories, enhance the efficiency of the authorization process, and reduce unnecessary trade barriers, while ensuring food safety.

A final draft agreed upon by the EU institutions resulted in the preparation of the new Novel Food Regulation (EU) 2015/2283, which entered into force on 1 January 2018. The new Regulation centralized the authorization procedure at EU level with deadlines imposed on certain stages and the immediate involvement of EFSA in the risk assessment process. The applicant-based authorization was replaced by a generic authorization to avoid duplication of work and an Union list comprising all authorized novel foods was established. Furthermore, the definition and categories of Novel Foods were revised and a procedure for the determination of a Novel Food status was implemented. To simplify the authorization of traditional foods safely used in third countries a faster and structured notification process was introduced. For the sake of clarity and to facilitate understanding, all legislation and guidance documents concerning the new Novel Food Regulation (EU) 2015/2283 are listed in Table 1.

Revision of the Novel Food Categories

The Article 3 (2a) of the Regulation (EU) 2015/2283 contains the fundamental definition of Novel Food meaning " [. . .] any food that was not used for human consumption to a significant degree within the Union before 15 May 1997, irrespective of the dates of accession of Member States to the Union, and that falls under at least one of the following categories." The 15 May 1997 refers to the date of entry into force of the former Novel Food Regulation and was established in the new Novel Food Regulation to ensure continuity between both Regulations. The novel food categories covered by Regulation (EU) 2015/2283 comprise now ten categories of which four already existing categories have been revised, clarified, and updated, and whereas six further categories have been newly added (Table 2).

As under Regulation (EC) No 258/97, food enzymes (falling within the scope of Regulation (EC) No 1332/2008 (2008)), food additives (falling within the scope of Regulation (EC) No 1333/2008 (2008)), food flavorings (falling within the scope of Regulation (EC) No 1334/2008 (2008)), and extraction solvents used or intended to be used in the production of foodstuffs or food ingredients are excluded. However, some foods may be considered as novel foods if employed for a different intended use or manufactured in a different way. One example is Lycopene which is used

Table 1 Overview over legislation and guidance documents related to the Novel Food Regulation (EU) 2015/2283

Regulation/Guidance	Title
Regulation (EU) 2015/2283	**Regulation** (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 **on novel foods**, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001
Implementing Regulation (EU) 2018/456	Commission Implementing Regulation (EU) 2018/456 of 19 March 2018 on the **procedural steps of the consultation process for determination of novel food status** in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods
Implementing Regulation (EU) 2017/2468	Commission Implementing Regulation (EU) 2017/2468 of 20 December 2017 laying down **administrative and scientific requirements concerning traditional foods from third countries** in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods
Implementing Regulation (EU) 2017/2469	Commission Implementing Regulation (EU) 2017/2469 of 20 December 2017 laying down **administrative and scientific requirements for applications referred to in Article 10** of Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods
Implementing Regulation (EU) 2017/2470	Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 **establishing the Union list** of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods
Implementing Regulation (EU) 2018/1023	Commission Implementing Regulation (EU) 2018/1023 of 23 July 2018 **correcting Implementing Regulation (EU) 2017/2470 establishing the Union list** of novel foods
EFSA guidance on applications for authorization	Guidance on the **preparation and presentation of an application for authorization** of a novel food in the context of Regulation (EU) 2015/2283 Administrative guidance on the **submission of applications for authorization of a novel food pursuant to Article 10** of Regulation (EU) 2015/2283
EFSA guidance on notifications and applications of traditional food	Guidance on the **preparation and presentation of the notification and application for authorization of traditional foods from third countries** in the context of Regulation (EU) 2015/2283
Information and guidance document	Guidance on human **consumption to a significant degree**

Table 2 Novel Food categories under the Regulation (EU) 2015/2283 and the former Regulation (EC) No 258/97 (Food categories newly added in Regulation (EU) 2015/2283) are given in red color)

Regulation (EU) 2015/2283		Regulation (EC) No 258/97 (2009)*
i	Food with a new or intentionally modified molecular structure, where that ***structure was not used as, or in, a food*** within the Union before 15 May 1997	Foods and food ingredients with a new or intentionally **modified primary molecular structure**
ii	Food **consisting of, isolated from** or ***produced*** from microorganisms, fungi or algae	Foods and food ingredients **consisting of** or **isolated from microorganisms, fungi or algae**
iii	Food **consisting of, isolated from** or **produced from material of mineral origin**	
iv	Food **consisting of, isolated from** or ***produced from plants or their parts, except*** when the ***food has a history of safe food use within the Union*** and is consisting of, isolated from or produced from a plant or a variety of the same species obtained by: — traditional propagating practices which have been used for food production within the Union before 15 May 1997; or — non-traditional propagating practices which have not been used for food production within the Union before 15 May 1997, where those practices do not give rise to significant changes in the composition or structure of the food affecting its nutritional value, metabolism or level of undesirable substances	Foods and food ingredients **consisting of** or **isolated from plants** and **food ingredients isolated from animals**, except for foods and food ingredients obtained by traditional propagating or breeding practices and having a history of safe food use
v	Food **consisting** of**, isolated from** or ***produced from animals*** *or* ***their parts***, except for animals obtained by traditional breeding practices which have been used for food production within the Union before 15 May 1997 and the food from those animals has a history of safe food use within the Union	
vi	Food consisting of, isolated from or produced from **cell culture or tissue culture** derived from animals, plants, micro-organisms, fungi or algae	
vii	Food ***resulting from*** **a production process not used for food production within the Union** before 15 May 1997, which gives rise to significant changes in the composition or structure of a food, affecting its nutritional value, metabolism or level of undesirable substances	Foods and food ingredients to which has been applied a **production process not currently used**, where that process gives rise to significant changes in the composition or structure of the foods or food ingredients which affect their nutritional value, metabolism or level of undesirable substances.
viii	Food consisting of **engineered nanomaterials** as defined in point (f) of this paragraph	
ix	**Vitamins, minerals and other substances** used in accordance with Directive 2002/46/EC, Regulation (EC) No 1925/2006 or Regulation (EU) No 609/2013, where: — a **production process not used for food production** within the Union before 15 May 1997 has been applied as referred to in point (a) (vii) of this paragraph; or — they **contain or consist of engineered nanomaterials** as defined in point (f) of this paragraph	
x	Food used exclusively in food supplements within the Union before 15 May 1997, where it is **intended to be used in foods other than food supplements** as defined in point (a) of Article 2 of Directive 2002/46/EC	

for technical purposes as food additive (E160d) and falls within the scope of Regulation (EC) No 1333/2008, whereas any other food uses of this product have to be authorized pursuant to the Novel Food Regulation (e.g., purified lycopene from tomatoes [*Lycopersicon esculantum* L.]).

It is worth noting that two more categories (foods and food ingredients containing or consisting of genetically modified organisms within the meaning of Directive 90/220/EEC; foods and food ingredients produced from, but not containing, genetically modified organisms) existed in the original version of Regulation (EC) No 258/97 (version 14.2.97) but were deleted due to an amendment by Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. Thus food products containing, consisting, or produced from genetically modified organisms (GMOs) do not anymore fall within the scope of the Novel Food Regulation but are regulated under the separate, specific Regulation (EU) No 1829/2003 (2003) on genetically modified food and feed since 2003.

Furthermore, foods consisting of/isolated from/produced from plants or their parts are now listed separately from food consisting of/isolated from/produced from animals or their parts. While Regulation (EC) No 258/97 comprised a legal uncertainty for whole insects as the applicable food category was defined as "food ingredients isolated from animals," the situation regarding the authorization of insect-based products, whole insects, parts of insects, or food ingredients isolated from insects as Novel Foods, if no history of consumption is approved, is now clarified. Consequently, a number of applications under the new Novel Food Regulation are now dedicated to insects, e.g., the house cricket (*Acheta domesticus*), the larvae of the lesser mealworm (*Alphitobius diaperinus*), the larvae of the black soldier fly (*Hermetia illucens*), the male pupae of the honey bee (*Apis mellifera*), the tropical house cricket (*Gryllodes sigillatus*), the migratory locust (*Locusta migratoria*), or the larvae of the mealworm beetle (*Tenebrio molitor*).

Considering the expansion of nanotechnologies used in the food industry, food containing or consisting of engineered nanomaterials was also included as Novel Food along with a definition of engineered nanomaterial as "intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts."

Determination of Novel Food Status

Article 4 of the Novel Food Regulation (EU) 2015/2283 lays down basic principles on the procedure for the determination of the novel food status and requires food business operators to verify whether the food which they intend to place on the Union market falls within the scope of that Regulation or not.

Accordingly, the Novel Food catalog may serve as an indicative source of information on the history of use of the product. This nonexhaustive list maintained by the European Commission is based on information provided by the Member States and gives an orientation on whether a product of animal and plant origin or other substances will need an authorization under the Novel Food Regulation. Nevertheless, the food business operator should take into account specific national

regulations that may restrict the use of the product as food (e.g., considered as medicine), even in the case products have a history of use for human consumption in some Member States.

In case of uncertainties regarding the Novel Food status, the food business operator can consult the Member State, where the food is first intended to be marketed. This recipient Member State then determines, based on necessary information provided by the food business operator, whether or not a food was used for human consumption to a significant degree within the Union before 15 May 1997. In order to determine the Novel Food status the recipient Member state may also consult other Member States and the European Commission. Within 4 months after verifying the validity of the consultation request the recipient Member State concludes on the Novel Food status of the food and the outcome is made publicly available by the European Commission on the Commission's website.

More detailed guidance on the consultation process can be found in Commission Implementing Regulation (EU) 2018/456 (2018). In addition to determing the Novel Food status according to Article 4 of Regulation (EU) 2015/2283, Article 5 authorizes the European Commission to decide, on its own initiative or upon a request by a Member State, on the novel food status of a particular food.

A key element in the determination of a novel food status is the history of human consumption to a significant degree within the Union before 15 May 1997. To address the sometimes difficult question of "human consumption to a significant degree" an information and guidance document is available on the European Commission's webpage (EC (European Commission) 2020) laying down specific criteria that should be considered. In addition, the Novel Food Regulation (EU) 2015/2283 clarifies that the history of consumption of a food as food supplement before 15 May 1997 is not relevant for the evaluation of whether a food was used for human consumption to a significant degree within the Union before 15 May 1997. However, foods that were exclusively used as food supplements before 15 May 1997 are not considered to be novel if they are intended to be used as or in food supplements after this date.

More than 30 consultation requests under Article 4 have already been decided until now (March 2020), including among others an application for consultation to determine the Novel Food status of berries of *Aristotelia chilensis* (Maqui berries). Here, it was decided by the German Federal Office for Consumer Protection and Food Safety that Maqui berries are considered to be not novel for use as or in food supplements due to an authorization of Maqui berries as or in food supplements in Italy. Apart from that, Maqui berries have a history of being used for the coloring of wine in the EU. However, this was not approved to constitute a "consumption to a significant degree." As there were no evidences for the use of Maqui berries for nutritional purposes, it was decided that they have to be considered as Novel Food when used for food uses other than food supplements.

Traditional Foods from Third Countries

A further substantial specification was introduced by defining specific rules for novel foods with a history of safe food use in a third country. In order to be acknowledged as a "traditional food from a third country" the safety of the food

has to be confirmed with compositional data along with a history of safe consumption by a significant number of people in at least one-third country for at least 25 years. Furthermore, the food has to be derived from primary production as defined in Regulation (EC) No 178/2002. For example, a juice derived from an exotic fruit not consumed in the European Union before 15 May 1997, but having been part of a regular diet in a third country, would be considered as a "traditional food." If this is the case, a faster and simplified notification procedure is set out.

However, this applies only to novel foods belonging to the categories "microorganisms, fungi, or algae," "animals or their parts," "plants or their parts," and "cell or tissue cultures derived from animals, plants, microorganisms, fungi, or algae" (for detailed description see food categories ii, iv, v, and vi in Table 2). The Articles 14 to 20 of the Regulation (EU) 2015/2283 lay down the specific rules and requirements for this category of Novel Foods (see section "Notification and Application for Authorization of Traditional Foods from Third Countries").

Up to now (March 2020), nine notifications of traditional foods from countries outside the EU have been submitted. All of these notifications concern foods derived from plants. Three of the notified foods have already been authorized as traditional foods from third countries: berries of *Lonicera caerulea* L. (Commission Implementing Regulation (EU) 2018/1991 1991), decorticated grains of *Digitaria exilis* (Commission Implementing Regulation (EU) 2018/2016 2018), and syrup from *Sorghum bicolor* (L.) Moench (Commission Implementing Regulation (EU) 2018/2017 2018). Additionally, two notifications concerning the same traditional food, namely fruit pulp from the cocoa plant *Theobroma cacao* L. (Commission Implementing Regulation (EU) 2020/206 2020), have recently been authorized as well. Regarding the traditional foods leaf powder from *Moringa stenopetala* (EFSA (European Food Safety Authority) 2019a) and powder or juice concentrate of berries of *Aristotelia chilensis* (Maqui berries) (EFSA (European Food Safety Authority) 2019b), EFSA has raised safety objections to the placing on the market within the EU. In contrast, no safety objections were submitted by EFSA concerning an infusion from coffee leaves (*Coffea arabica* L. and/or *Coffea canephora* Pierre ex A. Froehner) as a traditional food from a third country (EFSA (European Food Safety Authority) 2020). For roasted sacha inchi seeds the notification process is still ongoing and the safety assessment by the EFSA is not yet published.

Union List of Generic Authorized Novel Foods

Once a food is authorized as a novel food, or a traditional food from a third country is successfully notified, it is included in the Union list of Novel Foods authorized to be placed on the market within the Union. According to Article 7 of the Novel Food Regulation (EU) 2015/2283, the European Commission only authorizes and includes a novel food in the Union list if it is in accordance with the following conditions:

(a) The food does not, on the basis of the scientific evidence available, pose a safety risk to human health.
(b) The food's intended use does not mislead the consumer, especially when the food is intended to replace another food and there is a significant change in the nutritional value.
(c) Where the food is intended to replace another food, it does not differ from that food in such a way that its normal consumption would be nutritionally disadvantageous for the consumer.

The Union list contains the name of the authorized novel food, the specification, the specified conditions of use regarding food category and maximum levels, additional specific labeling requirements, and other requirements like post-market monitoring requirements which may be imposed by the European Commission for food safety reasons.

In addition to the authorized Novel Foods under Regulation (EU) 2015/2283, the Union list also contains all Novel Foods authorized and notified under the former Novel Food Regulation (EC) No 258/97. These novel foods were included in the Union list established through Implementing Regulation (EU) 2017/2470 (2017). On 23 July 2018, the Union list was corrected by replacing the Annex to Implementing Regulation (EU) 2017/2470 with the Annex to Implementing Regulation (EU) 2018/1023 (2018) due to the inclusion of newly authorized novel foods and the correction of a number of errors and omissions regarding existing entries of already authorized novel foods.

Once a novel food is included in the Union list, the authorization is generic meaning any food business operator can directly market their products following the conditions and specifications set out in Implementing Regulation (EU) 2018/1023. If that is not the case, food business operators may request a so-called extension of a Novel Food authorization, and therefore have to submit a new application to the European Commission for adding, removing, or changing the conditions, specifications, or requirements set out in the Union list.

The Union list will be updated regularly by the European Commission when a novel food is added or removed from the list, or when the conditions of commercialization have changed.

The concept of generic authorizations replaces the previously valid principle of the former Regulation (EC) No 258/97 that foods being substantially equivalent to existing foods were not subject to authorization. To protect the applicant's investment and to promote research, development, and innovation within the agri-food sector, applicants that submit newly developed scientific evidence and proprietary data can request data protection for 5 years. Subsequent applicants cannot benefit from the application during this period unless they obtain authorization for the novel food without reference to the protected data or with the agreement of the initial applicant. Where data protection is granted, the Union list specifies it, and indicates that during the period of data protection the novel food is authorized for placing on the market within the Union only by the initial applicant.

Transitional Measures

Foods which were lawfully placed on the market prior to 1 January 2018 and did not fall within the scope of the former Regulation (EC) No 258/97, but are subject to the new Novel Food Regulation (EU) 2015/2283, are covered by transitional provisions laid down in Article 35 of this Regulation. Provided that an application for authorization of a Novel Food or a notification of a traditional food from a third country has been submitted by 1 January 2019, these foods can continue to be placed on the market until an outcome of the authorization procedure has been decided. In addition, the transitional measures also regulate that applications for placing a novel food on the market under the former Novel Food Regulation (EC) No 258/97 not finally decided by 1 January 2018 are treated as applications under the new Regulation (EU) 2015/2283.

Authorization Procedure in the EU

Food business operators, who intend to introduce a "new food" into the EU market, have to determine in advance whether their food is subject to the Novel Food Regulation (EU) 2015/2283. This means they have to check if the food falls under the definition of Novel Food and into one of the specified novel food categories as presented in section "Revision of the Novel Food Categories." The novel food catalog (section "Determination of Novel Food Status") may serve as an orientation in addressing the question of whether a food has to be regarded as a novel food or not. Food business operators may also submit a formal request to a Member State according to Article 4 to clarify the status of the food. If the "new food" is indeed regarded as a novel food, it will need an authorization under Regulation (EU) 2015/2283, either as an authorization of a novel food or as a notification of a traditonal food from a third country.

Authorization Procedure for Novel Foods

To ensure an EU-wide harmonized authorization and safety assessment of novel foods the new Novel Food Regulation introduced a centralized autorization procedure managed by the European Commission. The different steps of the authorization procedure are laid down in chapter III of Regulation (EU) 2015/2283 in Articles 10 to 13.

Before placing a novel food on the market within the European Union for the first time, the applicant, as defined in Article 3 (2a) of Regulation (EU) 2015/2283, has to directly submit an application for authorization to the European Commission in line with the requirements of Article 10 of the Regulation using the "e-submission"-system (Fig. 1). The application dossier should be in accordance with Commission Implementing Regulation (EU) 2017/2469 (2017) which lays down specific rules for the implementation regarding the administrative and scientific requirements for applications as well as the transitional measures.

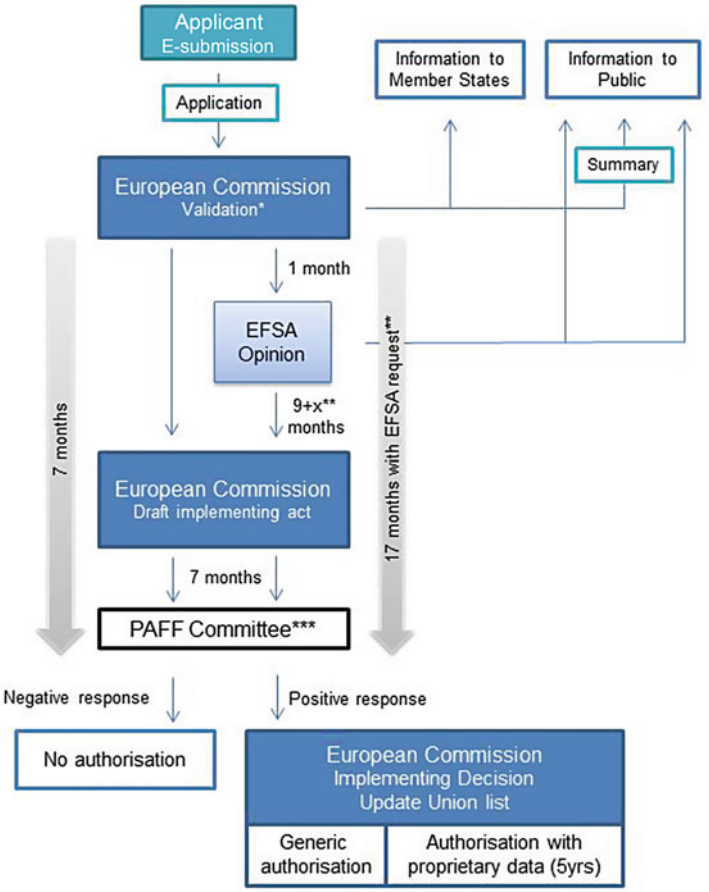

Fig. 1 Authorization procedure according to Novel Food Regulation (EU) 2015/2282. * Verification of validity + possible consultation with EFSA; ** Time period may be extended when EFSA requests additional information from the applicant; *** Standing Committee on Plants, Animals, Food and Feed

Having received the application, the European Commission verifies the validity of the application dossier. If the European Commission requests the opinion of the European Food Safety Authority (EFSA) it forwards the valid application without delay and within 1 month of verifying the validity of the application to EFSA which carries out a scientific assessment of the safety of the proposed novel food within 9 months from the date of receipt of the valid application. If EFSA requests additional information from the applicant, the 9-month period may be extended. After EFSA has forwarded its opinion to the European Commission, to the Member States and, where applicable, to the applicant, the Commission submits a draft implementing act for the authorization of the novel food and updating of the Union list to the Standing Committee on Plants, Animals, Food and Feed (PAFF Committee), composed of

representatives of the Member States, within 7 months from the date of the publication of the EFSA opinion. In case the European Commission has not requested an opinion from EFSA, the 7-month period starts from the date on which a valid application is received by the Commission. Once the implementing act receives a positive response from the PAFF Committee and is adopted and published by the European Commission, the authorized novel food can be placed on the market within the European Union and the Union list is updated (see section "Union List of Generic Authorized Novel Foods").

Risk Assessment Process

The risk assessment of novel foods aims to ensure that the requested Novel Food does not pose a safety risk to human health. In order to enable a comprehensive risk assessment of the novel food in question, the applicant should, according to Article 10 of the Novel Food Regulation (EU) 2015/2283, provide information on the name and description of the novel food, the production processes, the composition of the novel food, scientific evidence demonstrating that the novel food does not pose a safety risk to human health, the analysis methods (where appropriate), and a proposal for the conditions of intended use as well as a proposal for specific labeling requirements which do not mislead the consumer.

Upon request by the European Commission, the EFSA assesses the safety of the novel food under the proposed conditions of use. EFSA carries out its scientific risk assessment based on the dossier provided by the applicant and considers the following aspects (see EFSA guidance document 2016 (EFSA NDA Panel (European Food Safety Authority Panel on Dietetic Products, Nutrition and Allergies) 2016a)):

1. Whether the novel food concerned is as safe as food from a comparable food category already existing on the market within the Union.
2. Whether the composition of the novel food and the conditions of its use do not pose a safety risk to human health in the Union.
3. A novel food, which is intended to replace another food, does not differ from that food in such a way that its normal consumption would be nutritionally disadvantageous for the consumer.

EFSA has developed two guidance documents (EFSA NDA Panel (European Food Safety Authority Panel on Dietetic Products, Nutrition and Allergies) 2016a; EFSA (European Food Safety Authority) 2018a) which provide scientific information and data needed for the safety assessment of novel foods and present a common format for the organization of the requested data. The guidance documents are intended to support applicants in the preparation of comprehensive applications and to facilitate an effective and consistent evaluation by EFSA in order to ensure a harmonized scientific assessment of novel foods. Apart from administrative data the application should contain technical and scientific data specific to the novel food which are of relevance for the risk assessment and provided in more detail in the following sections.

Introduction and Identity of the Novel Food

The novel food should be briefly introduced by describing the source, the principle of the production process, typical compositional features as well as the purpose and the intended use. For novel foods referring to chemical substances, polymers, or food consisting of, isolated from, or produced from material of mineral origin information on the identity of the novel food should comprise the chemical name, CAS number, or other identification numbers, synonyms, trade names and abbreviations, molecular and structural formulae, stereochemistry, molecular weigth as well as particle size, shape, crystal form, and distribution. For novel foods consisting of, isolated from, or produced from microorganisms, fungi, algae, plants/plant parts, animals/animal parts, or cell/tissue culture derived from animals/plants/fungi/algae, the identity should be substantiated by providing the scientific name, synonyms, common names, verification of the identity of plants, algae, and fungi according to internationally recognized databases and methodology, organ and tissue or parts used of the source organism, geographical origin, and laboratory or culture collection used as source. The characterization and identification of food consisting of engineered nanomaterials calls for a broader range of parameters. The required information regarding engineered nanomaterials are elaborated in detail in the EFSA guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain (EFSA SC (European Food Safety Authority Scientific Committee) 2018).

Production Process

To ensure that no safety concerns arise from the production process of the novel food, a comprehensive description of all information relevant to the production and processing of the novel food needs to be provided. This includes the detailed description of the processes involved in the production of the novel food. Information regarding the handling of sources, post-harvest handling of unprocessed foods, raw materials, and/or chemical substances used, processes employed to convert the raw materials into the product, specification of reaction conditions, purification methods, and identification of potential by-products, impurities, and contaminants should be provided. Furthermore, a description of operational limits and key parameters of the production process, measures concerning production control as well as quality and safety assurance, production flow charts indicating quality and safety control checks, and standardization criteria should be included.

One category of novel foods in the Novel Food Regulation (EU) 2015/2283 explicitly concerns the novelty of the employed production processes and refers to food resulting from a production process not used for food production within the Union before 15 May 1997. As the Regulation, in this regard, confines the term novel to production processes that do not significantly change the composition or structure of a food and do not affect the nutritional value, the metabolism or the level of undesirable substances, it is essential that all novel aspects of the production process as well as its effects on the bioavailibility, nutritional value and safety of the Novel Food in question are characterized in detail by the applicant.

Compositional Data

The composition of the novel food should be demonstrated by qualitative and quantitative data and should include information on physicochemical, biochemical, and microbiological characteristics. The data should contain the identification and quantification of substances of toxicological concern, impurities, by-products, residues, and contaminants. The analytical methods used should be validated and the provided data should include a description of the methods, the limit of detection and quantification as well as information on the accreditation of laboratories. To represent a certain range of variability, at least five representative and independently produced batches of the novel food should be analyzed. If the Novel Food refers to a single compound or fully chemically characterized mixtures thereof, compositional data should contain information on the identity and relative ratios of all components, the mass balance, identity tests used for the analyses, physicochemical properties as well as minimum purity. In contrast, complex mixtures or whole foods cannot be fully chemically characterized. Here, the qualitative and quantitative analysis should focus on the main constituents, proximate analyses, nutritionally relevant components, substances posing a possible concern to human health, and naturally or chemically derived components typically characteristic for the novel food. Analytical data on the composition of the novel food should be compared with data from literature.

Safety concerns may also arise from the storage and transport of the Novel Food. Therefore, the physicochemical, biochemical, and microbiological stability have to be tested and evaluated preferably on at least five representative and independently produced batches of the novel food.

Specification

The specification shall ensure that the product intended to be placed on the EU market complies with the analyzed and evaluated Novel Food. It should appropriately characterize the novel food by setting physicochemical, biochemical, and microbiological key parameters including the contents of nutritionally or biologically active compounds, contents of major groups of food constituents, purity, limits for impurities, and degradation products as well as maximum levels for chemical and microbiological contaminants.

History of the Novel Food and/or of Its Source

Information on the experience gained with a Novel Food regarding the previous exposure and use of a novel food outside the EU is of high relevance for the risk assesment. This includes data on the use of the Novel Food outside the EU as well as for nonfood purposes. Relevant aspects to assess the history of use are the extent of use, the characterization of the population groups which have consumed the Novel Food, the role of the novel food in the diet of these population groups, specific information regarding the handling and preparation of the food as well as precautions regarding its preparation and use. Furthermore, information on human studies about relevant safety aspects of the novel food in question resulting from a thorough and comprehensive literature review should be provided by the applicant. In general,

data concerning the history of use of a novel food should not be limited to the novel food itself but, in addition, should also include information on the composition, production, and experience from use of other products derived from the same source (e.g., a certain plant) as the novel food.

Proposed Use and Use Levels and Anticipated Intake

An essential part of risk characterization is the exposure assessment. To estimate the intake of the novel food in question, information on the use levels of the novel food and data on the food consumption are needed. The use and use levels are proposed by the applicant. This requires to specify the target population, the form of uses, the food categories in which the Novel Food is intended to be used, the proposed maximum use levels, and concentrations in the final product intended to be consumed and the proposed daily intakes. It also needs to be clarified whether the novel food is meant to replace another food. Food consumption data should be retrieved from representative databases like the EFSA Comprehensive European Food Consumption Database (EFSA (European Food Safety Authority) 2011a) or national dietary surveys. Based on these information the anticipated mean and high daily intakes are estimated taking into account different population groups, combined comsumption of all the food categories the Novel Food is proposed to be used in, and different consumption scenarios. Additionally, it might occur that a novel food also has other dietary sources like being a natural constituent in food. In this case, the combined exposure from the novel food and the background diet must be taken into consideration and the extent of the additional intake of the novel food in relation to the overall intake should be assessed. Even nondietary sources like cosmetics or pharmaceuticals might be relevant and considered in the overall exposure assessment. As the compositional analysis of the novel food might also reveal undesirable compounds present in the novel food, the intake assessment must also comprise such constituents. Based on the exposure assessment and all available data concerning the safety of the novel food, relevant precautions and restrictions regarding the use of the novel food should be specified.

Absorption, Distribution, Metabolism, and Excretion (ADME)

A comprehensive assessment of all relevant toxicological and nutritional aspects of the Novel Food also requires toxicokinetic information. Data on absorption, distribution, metabolism, and excretion should be preferably provided for all toxicologically and nutritionally relevant constituents of the Novel Food according to the principles outlined in the EFSA guidance for the evaluation of food additives (EFSA ANS PANEL (European Food Safety Authority Panel on Food Additives and Nutrient Sources added to Food) 2012). These principles describe a tiered approach to toxicokinetic testing. Tier 1 assesses whether the Novel Food or its breakdown products are absorbed. If it can be demonstrated that the absorption is negliable, omitting higher tiered toxicological studies may be scientifically justified. Tier 2 requires in vivo assessment of absorption, distribution, metabolism, and excretion as well as basic single dose toxicokinetic parameters after systemic exposure to a single dose. If indications for bioaccumulation like limited or slow excretion are observed,

tier 3 toxicokinetic testing is necessary to define toxicokinetic parameters following repeated administration. Requirements regarding ADME studies for Novel Foods consisting of engineered nanomaterials are specified in the EFSA guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain (EFSA SC (European Food Safety Authority Scientific Committee) 2018).

Nutritional Information

According to Article 7 of the Novel Food Regulation (EU) 2015/2283, a novel food that is intended to replace another food does not differ from that food in such a way that its normal consumption would be nutritionally disadvantageous for the consumer. The applicant has to demonstrate that the consumption of the novel food in question does not lead to nutritional disadvantages under the proposed conditions of use. To assess the novel food's impact on nutrient intake, comprehensive information about the nutritional composition regarding macro- and micronutrients and the bioavailability of the novel food are required. This also includes information regarding the presence of antinutritional constituents and any possible effects and interactions with nutrients. Furthermore, the proposed use levels and estimated exposure for nutritional and antinutritional factors should be considered and assessed in relation to available dietary intakes, tolerable upper intake levels, and relevant health-based guidance values. If the target population includes sensitive subpopulations with particular physiological or metabolic characteristics (e.g., infants, young children, pregnant, or lactating women), these population groups should be specifically considered in the nutritional evaluation.

Toxicological Information

As the safety of the novel food is assessed, the toxicological information should be derived from toxicological studies analysing the novel food in the form as it is intended to be placed on the market. Information on compounds having a structure similar to the Novel Food are suitable to assist in the safety assessment of the novel food in question. In general, the toxicological testing should follow international guidelines and principles of good laboratory practice. Similar to the evaluation of ADME data, toxicological testing should be carried out according to EFSA's tiered toxicity testing approach described for food additives (EFSA ANS PANEL (European Food Safety Authority Panel on Food Additives and Nutrient Sources added to Food) 2012). It adresses genotoxicity, subchronic toxicity, chronic toxicity, and carcinogenicity as well as reproductive and developmental toxicity.

Assessing the genotoxic and mutagenic potential in a chemical risk assessment aims for the identification of substances capable of causing heritable damage in humans, the prediction of potential genotoxic carcinogens when no carcinogenicity data are available, and elucidation of the specific mechanism of actions of chemical carcinogens. Following a stepwise approach a series of in vitro assays (bacterial reverse mutation assay (OECD (Organisation for Economic Co-Operation and Development) 1997), in vitro mammalian cell micronucleus test (OECD (Organisation for Economic Co-Operation and Development) 2016a)) are initially

recommended to evaluate the induction of gene mutation and chromosomal abberations (clastogenicity/aneuploidy). In the case of positive results, further approaches are recommended including substitution and completion of in vitro assays with other appropriate in vitro and in vivo assays (e.g., in vivo micronucleus test (OECD (Organisation for Economic Co-Operation and Development) 2016b), in vivo Comet assay (OECD (Organisation for Economic Co-Operation and Development) 2016c), and transgenic rodent assay (OECD (Organisation for Economic Co-Operation and Development) 2013)). The evaluation of test results and the selection of follow-up assays should always consider all available relevant data of the substance including chemical reactivity, bioavailability, metabolism, toxicokinetics, target organ specificity, and endpoints. For novel foods representing complex mixtures or whole foods which naturally encompass a multitude of different compounds, it is recommended to focus on toxicologically relevant constituents of the Novel Food.

In addition to genotoxicity, the safety assessment of a Novel Food requires the evaluation of subchronic toxicity. Subchronic toxicity studies aim to provide information on the affected target organs and tissues, the type, extent, and severity of any effects as well as on dose-response relationships including the determination of reference points/points of departure like the relevant benchmark dose lower confidence limit (BMDL) or the no observed adverse effect level (NOAEL). Furthermore, they should assist in estimating appropriate dose levels for chronic toxicity studies and provide information regarding the need for additional in-depth investigation of particular effects and endpoints, e.g., neurotoxic effects, immunological effects, reproductive organ effects, or endocrine-mediated effects. Subchronic toxicity data should be derived from a repeated dose 90-day oral toxicity study in rodents (OECD (Organisation for Economic Co-Operation and Development) 2018a) modified to include additional parameters to assess endocrine activity as described for repeated dose 28-day oral toxicity studies in rodents (OECD (Organisation for Economic Co-Operation and Development) 2008). In absence of systemic availability, analyses should focus on pathological and physiological effects in the gastrointestinal tract.

Critical findings resulting from preceding genotoxicity tests and subchronic toxicity studies may raise the need for conducting a chronic toxicity (OECD (Organisation for Economic Co-Operation and Development) 2018b) and a carcinogenicity study (OECD (Organisation for Economic Co-Operation and Development) 2018c). Both studies are preferably performed in rats for a duration of 12 months (chronic tocxicity) or 24 months (carcinogenicity). Alternatively to conducting two seperate studies, it is also possible to perform a more time and cost-effective combined chronic toxicity/carcinogenicity study (OECD (Organisation for Economic Co-Operation and Development) 2018d).

Reproductive toxicity studies aim to reveal effects on male and female fertility, on the female's ability to carry pregnancy to term and on maternal lactation and care of the young. Regarding effects on the offspring, they provide information on the prenatal and postnatal survival, growth, functional and behavioral development, and reproductive capacity. Additionally, the studies enable the histological identification of important target organs for toxicity in parents and offspring. Prenatal

developmental toxicity studies provide information on lethal, teratogenic, or other toxic effects on the embryo and fetus. The need for reproductive and developmental toxicity studies has to be evaluated based on available toxicokinetic and toxicity information. In the case of proven or suspected systemic availability of a substance or if the repeated dose 90-day oral toxicity study indicates any effects on reproductiove organs or parameters, reproductive and developmental toxicity testing is required. Recommended testing studies are a prenatal developmental toxicity study in the rabbit (OECD (Organisation for Economic Co-Operation and Development) 2018e) and an extended one-generation reproduction toxicity study in the rat (OECD (Organisation for Economic Co-Operation and Development) 2018f).

Furthermore, relevant information regarding the safety of a novel food can be derived from availiable human studies. Such studies may assist in evaluating potential adverse effects and in demonstrating the safety of the novel food under the proposed conditions of use.

There is an increasing interest in the consumption of insects as novel foods. The production and consumption of insects as food and feed may be associated with specific microbiological, chemical, and environmental hazards. These insect-related risks have been comprehensively identified and evaluated by EFSA and should be carefully considered in the safety assessment of insects as novel food (EFSA SC (European Food Safety Authority Scientific Committee) 2015).

Novel Foods may also be composed of, isolated from, or produced from microorganisms. Thus, ensuring that the novel food in question does not pose a risk to human health requires a microbiological safety assessment. This includes taxonomic classification at the species level and strain characterization at the genomic sequence level for the detection of potential virulence-related genes, antibiotic resistances, and their potential horizontal transfer. Additional potentially adverse genotypic and phenotypic characeteristics and features should be evaluated as well. If a microorganism has a history of safe use and is assigned to a group of microorganism with a qualified presumption of safety (QPS) status, the requirements for the safety evaluation are reduced to complying with the criteria and qualifications specified in the QPS list (EFSA (European Food Safety Authority) 2008) and the risk assessment of antimicrobial resistance (EFSA FEEDAP Panel (European Food Safety Authority Panel on Additives and Products or Substances used in Animal Feed) 2012). A QPS status is granted for taxonomic groups posing no safety risk or where safety concerns can be defined and excluded.

Regarding the toxicological safety assessment of novel foods consisting of engineered nanomaterials, the specific data requirements and principles are outlined in the EFSA guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain (EFSA SC (European Food Safety Authority Scientific Committee) 2018).

Allergenicity

To ensure the safety of a novel food, the allergenic potential of the novel food needs to be evaluated. As most food allergens are protein-based, all novel foods containing proteins are assumed to potentially elicit allergic reactions. To assess the allergenic

potential information regarding sensitization, studies on allergenicity and case reports concerning allergic reactions are needed. Investigation of allergenicity may include data on structural aspects of the proteins or peptides, detection of cross-reactive IgE antibodies, skin prick testing, or double-blind placebo-controlled oral food challenges. Evidence for potential allergenic reactions of a novel food requires risk management measures like restriction and labeling of the novel food to protect potentially affected consumers.

Notification and Application for Authorization of Traditional Foods from Third Countries

The new Novel Food Regulation (EU) 2015/2283 introduces a faster and more appropriate notification procedure for traditional foods from non-EU countries with a demonstrated safe history of use of at least 25 years. The specific requirements applying to traditional foods from third countries are set out in Article 14 to 20.

Before placing the food on the market within the European Union as traditional food, the applicant has to directly submit an online notification for authorization to the European Commission (Fig. 2) which has to be in accordance with the requirements in Article 14 of the Regulation (EU) 2015/2283 and Commission Implementing Regulation (EU) 2017/2468 (2017) setting out administrative and scientific requirements for applications for authorizations of traditional foods from third countries. Within 1 month after verifying the validity of the notification, the European Commission forwards the notification to the Member States and EFSA. If EFSA and Member States do not raise duly reasoned safety concerns regarding the placing on the market within the Union of the traditional food within a period of 4 months, the traditional food is authorized by the European Commission and the Union list is updated. However, in case of duly reasoned safety objections submitted by EFSA or Member States, the European Commission neither authorizes the traditional food concerned nor updates the Union list. Instead, the applicant may submit an application for the authorization of traditional foods from third countries to the European Commission following the requirements of Article 16 of Regulation (EU) 2015/2283. Here, in addition to the information already requested for the notification procedure for traditional foods from third countries according to Article 14, the applicant is also required to provide specific data related to the duly reasoned safety objections raised by EFSA or Member States.

The application for the authorization of traditional foods from third countries is forwarded without delay to EFSA and made available to the Member States by the European Commission. EFSA is requested by the European Commission to review the application and assess the safety of the traditional food from a third country within 6 months. The 6-month period may be extended if EFSA requests additional information from the applicant. After receiving EFSA's opinion on the application within 3 months the European Commission submits to the PAFF Committee a draft implementing act authorizing the placing on the market within the Union of the traditional food from a third country. When the authorization is granted, the Union

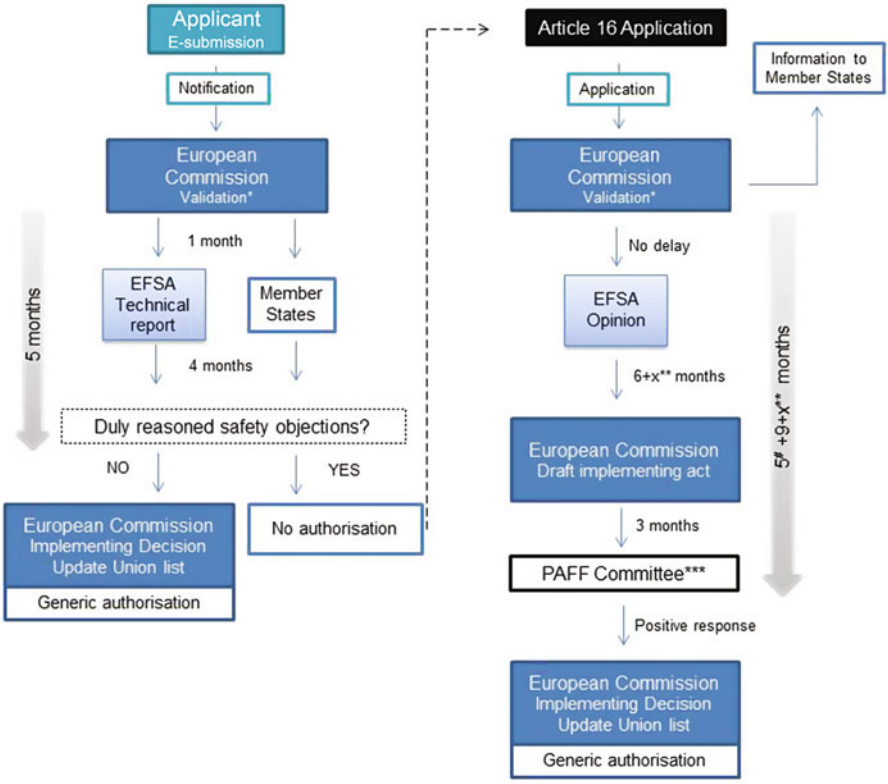

Fig. 2 Notification procedure and application for authorization of traditional foods from third countries according to Novel Food Regulation (EU) 2015/2282. * Verification of validity + possible consultation with EFSA; ** Time period may be extended when EFSA requests additional information from the applicant; *** Standing Committee on Plants, Animals, Food and Feed; # Time period due to notification procedure

list will be updated. Overall, the time frame for the EFSA opinion and the European Commission decision are somewhat shorter for an application for the authorization of a traditional food from a third country compared to the regular authorization procedure of Novel Foods, though the process is still complex and time-consuming for the applicant.

Safety Assessment Procedure for Traditional Foods from Third Countries

Before a traditional food from a third country pursuant to Regulation (EU) 2015/2283 and Commission Implementing Regulation (EU) 2017/2468 (2017) can be placed on the market within the EU, it must be subjected to a premarket safety assessment based on a history of safe food use. Notifications according to Article 14 of Regulation (EU) 2015/2283 should contain sufficient information and scientific

documentation for the European Commission to verify the validity and enable Member States and EFSA to evaluate the history of safe use of the traditional food in at least one country outside of the EU for a period of at least 25 years. These information should include the name, description, and composition of the traditional food, the country/countries of origin, documented data demonstrating the history of safe food use in a third country as well as a proposal for the conditions of intended use and for specific labeling requirements not misleading the consumer.

In order to support applicants in providing the entire type and quality of information relevant to conclude whether there are reasoned safety objections, EFSA provided a scientific and technical guidance document for the preparation and presentation of the notification dossier (EFSA NDA Panel (European Food Safety Authority Panel on Dietetic Products, Nutrition and Allergies) 2016b). As indicated in this guidance document, it is the duty of the applicant to provide all the available and reliable data that are pertinent to the safety of the traditional food.

Consistent with application dossiers of novel foods according to Article 10 of Regulation (EU) 2015/2283, the notification dossier needs to contain information on the identity of the traditional food and reliable data on the respective production process, the composition and specifications of the traditional food as described in sections "Introduction and Identity of the Novel Food," "Production Process," "Compositional Data," and "Specification." In addition, the applicant has to demonstrate the experience of continued use of the traditional food and has to propose conditions of use for the EU market. In contrast to the evaluation of novel foods, the applicant is not specifically requested to provide specific information or perform specific studies on toxicokinetic, toxicological, nutritional, and allergenic properties of the traditional food (see Table 3). However, if such data are available they should be provided to ensure a comprehensive risk assessment of the traditional food covering all relevant safety aspects.

Data from the Experience of Continued Food Use in the Third Country

A central point in the safety assessment of a traditional food is the experience of continued use of the traditional food for at least 25 years in the third country. Data should cover a description of the extent of use including data on production (e.g., place, volume per year) and geographical areas along with information on intake levels and intake estimates. For example, in the case of notifying the placing on the market of berries of *Lonicera caerulea* L. (Haskap berries) as a traditional food from a third country, the applicant provided amongst others references to document the annually average consumption per person over the past 30 years in Hokkaido (Japan) (EFSA (European Food Safety Authority) 2018b).

In addressing the continued food use, information on the specific population group(s) of consumers (e.g., general population or subpopulations defined by specific criteria like age and ethnic background) along with the group size should be provided by the applicant. The role of the traditional food in the diet should also be documented including consumption pattern and frequency (e.g., consumed in the form of beverages or as food ingredient in processed foods). It may be conducive to supply data on how the intake of the traditional food contributes to the total intake of

Table 3 Data requirements of the application/notification dossier for risk assessment of novel foods and traditional foods from third countries (EFSA NDA Panel (European Food Safety Authority Panel on Dietetic Products, Nutrition and Allergies) 2016a, b)

Application for authorization of novel foods	Notification for authorization of traditional foods from third countries
Identity of the novel food	Identity of the traditional food
Production process	Production process
Compositional data	Compositional data
Specifications	Specifications
History of use of the novel food and/or of its source	Data from experience of continued use
Proposed uses and use levels and anticipated intake	Proposed conditions of use for the EU market
Absorption, distribution, metabolism, and excretion	
Nutritional information	
Toxicological information	
Allergenicity	

macro- and micronutrients of the population. Additionally, the traditional manufacture, preparation, preservation, packaging, or storage should be described.

Precautions regarding preparation and handling of the food as well as restrictions of use (e.g., for specific subpopulations like children) should be provided along with any kind of treatments or methods leading to the reduction of toxicological relevant and antinutritional substances or improving the digestibility of the food. Furthermore, any available human data related to the safety of the traditional food should be reported including clinical trails, observational studies, and information from case and surveillance reports. Additionally, data on specific and typical components of the traditional food or on similar foods from related sources as well as other important data (e.g., animal toxicity studies and nonfood use in medicine) should be adressed to support a conclusion on the history of safe use of the notified traditional food.

Proposed Conditions of Use for the EU Market

In order to place on the market within the EU a traditional food from a third country, the notification should clearly and specifically define the target population intended to consume the traditional food. It is particularly important to provide specific information about the proposed uses und use levels to evaluate whether the traditional food may pose a risk for human consumption. This includes the form of uses and clearly defined food categories (preferentially by following the EFSA food classification system (EFSA (European Food Safety Authority) 2011b)). It needs to be clarified whether the traditional food is supposed to replace another food and that the consumption of the traditional food is not nutritionally disadvantageous for the consumer. The proposed maximum use levels and concentrations in the final

product as well as the proposed daily intake levels for different subgroups classified by age and gender should, if applicable, also be indicated.

Regarding restrictions of use of the traditional food the (sub)groups of the population which should avoid the consumption should be defined and any other restrictions and precautions related to the handling, preparation, and consumption should be described along with any effects of potential overconsumption on population or subgroups of population.

Cross-References

- ▶ Exposure Scenarios in Toxicology
- ▶ Microbiome Product Toxicology: Regulatory View on Translational Challenges
- ▶ Risk Assessment of Food Additives
- ▶ Risk Assessment of Genetically Modified Food and Feed

Notes

1. When the General Food Law Regulation (Regulation (EC) No 178/2002) entered into force in 2002 the tasks of the SCF were taken over by the EFSA.

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Microbiome Product Toxicology: Regulatory View on Translational Challenges

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Cécile F. Rousseau, Clara Desvignes, Fanny Kling, Emmanuelle M. Voisin, and Manfred Ruthsatz

Contents

C. F. Rousseau (✉) · E. M. Voisin
Voisin Consulting Life Sciences, Boulogne, Paris, France
e-mail: rousseau@voisinconsulting.com; voisin@voisinconsulting.com; vcparis@voisinconsulting.com

C. Desvignes
Voisin Consulting Life Sciences, Saint-Gregoire (Rennes), France
e-mail: desvignes@voisinconsulting.com

F. Kling
Voisin Consulting Life Sciences, Lausanne, Switzerland
e-mail: kling@voisinconsulting.com

M. Ruthsatz
Nutrition+HealthCARE, Vevey, Switzerland

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Abstract

The recent rise of science in the microbiome field has brought to light new opportunities of health technologies development. Microbiome-derived products are very diverse and encompass several product categories such as foods as well as medicinal products. While some guidance and recommendations are being drawn for very specific microbiome applications, there is no consolidated regulatory framework applicable to all microbiome products. Any specific requirements should be carefully considered on a case-by-case basis in order to demonstrate the safety and efficacy of these new high potential healthcare solutions.

Specific challenges due to the nature of microbiome health products must be addressed for toxicology assessment and clinical translation. Colonization and proliferation processes for live microorganisms need to be controlled. Some solutions are already available for microbiome products development, such as dedicated nonclinical models, and emerging tools such as in silico modeling may potentially prove useful. This chapter aims to draw the regulatory landscape for microbiome-derived products prior to debating various nonclinical tests systems to evaluate their toxicology.

Keywords

Toxicology · Microbiome · Live-biotherapeutic products (LBP) · Gnotobiotic · Translation · Test system · Intestine

Introduction

The emergence of the microbiome field is leading toward new considerations in the areas of human health, well-being, and potentially even host personality traits (Johnson 2020), as well as the development of health technologies, especially for therapeutic purposes. As close as the relationship between microorganisms and their host may be, microbiota, as a "not-self" entity, cannot be considered the same way as human tissues.

The wide spectrum of therapeutic indications that may be the result of microbiome disorders is the consequence of the vast environment that microorganisms can inhabit in humans. More than 100 trillion microbes reside within tissues or biological fluids of different microbiota including but not limited to the gut, oral, skin, or vaginal microbiota. There is a lack of hindsight on these organisms' specificities and how to take them into account for therapeutic development. It is a common misconception that the commensal nature of the indigenous microorganisms guarantees their safety for the host. *Escherichia coli (E. coli)* is for instance an early colonizer of the intestinal tract, and it persists throughout the entire individual's life. However, certain *E. coli* strains are also among the most known bacterial pathogens.

Although microbiome-focused science is quite new, the odds are that a healthy microbiota-host relationship lies more in the balance of the overall microbiota composition rather than in the presence of specific strains and species. Thus, most microbial species and strains are not intrinsically beneficial or detrimental to the host's health. To make the most out of the very promising microbiome science for the development of alternative therapeutic solutions, accurate toxicology assessment is fundamental and constitutes a critical step toward clinical translation. Microbiome toxicology presents specific challenges which require the development of dedicated nonclinical models. Such investigations are as of now supported by the rise of the "omics" technologies (e.g., metagenomics, metataxomics, and metabonomics) (OECD 2017; Segal et al. 2019).

There is a paradigm shift as products known for decades and used in foods as technological aids are now investigated for their potential health benefits. They may be considered as foods with particular nutritional or health-related properties, or as medicinal products, defined by their mode of action, their therapeutic effect, and a dose defining their efficacy threshold.

In the fast-moving microbiome field, a key to success is to be aware of the extent and limitations of the current scientific knowledge in order to adopt and develop adequate solutions that will help ensure this emerging technology is explored and used in the most controlled possible way for the common good.

Regulatory Landscape for Microbiome Products

Lay Terminology and Regulatory Status

In the lay language, microbiome-health products are commonly designated as "prebiotics" and "probiotics." These terms have been used for decades with the first uses dating back to 1965 (Lilly and Stillwell 1965). In the past decade, the term "postbiotics" was coined to designate a new type of microbiome-related products.

There is to date no absolute consensus on these terms' definitions. Multiple interpretations have been proposed in the literature over the years. In addition, the microbiome field has grown considerably, and the development of new applications may render obsolete some of these definitions. Prebiotics and probiotics have often been considered as food components or foods themselves (Pineiro et al. 2008; Gibson and Roberfroid 1995; Patel and Denning 2013; El-Ashram et al. 2019). However, such wording may conflict with applications in the therapeutic field: the European Regulation indicates that "Foods" should not designate Medicinal products (EMA 2002b). Some definitions also suggest that these products are limited to gastrointestinal applications (Gibson and Roberfroid 1995; El-Ashram et al. 2019; Guarner and Schaafsma 1998; Parker 1974; LeBegue et al. 2020), while the benefits of prebiotics and probiotics on other tissues such as skin (Kaur and Rath 2019), brain (Heyck and Ibarra 2019), or vagina (Al-Ghazzewi and Tester 2016) are gaining more and more interest. It has also been suggested that prebiotics, probiotics, or

postbiotics could be limited to bacteria-related benefits (Gibson and Roberfroid 1995; Patel and Denning 2013; El-Ashram et al. 2019; Havenaar and Huis In't Veld 1992). Yet, the microbiome field is extending to other microorganisms such as yeasts (Massacci et al. 2019), fungi (Pareek et al. 2019; Huss and Raman 2020), or viruses (Pareek et al. 2019; Huss and Raman 2020).

For the purpose of this chapter, the following definitions will be considered for prebiotic, probiotic, and postbiotic:

- Prebiotic: Nonviable substance which, when administered in adequate amount, selectively promotes the growth, development, and/or activity of beneficial microorganisms hosted by the individual taking the product.
- Probiotics: Living microorganism(s), when administered in adequate amount, confer (a) health benefit(s) to the host (Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) 2002, 2006).
- Postbiotics: Nonviable substance produced by or derived from microorganisms, which, when administered in adequate amount, confer (a) health benefit(s) to the individual taking the product.

None of the "prebiotic," "probiotic," or "postbiotic" terms has ever been coined by the formal European or American regulatory lexicons. The FDA clarified in 2018 that probiotics are not defined as a regulatory product category (FDA 2018). In addition, there are no correspondences between these wordings and regulatory statuses. "Prebiotics," "probiotics," and "postbiotics" may encompass many official categories of products, both in the food or the therapeutic field.

In the food field, microbiome-related components are likely to be considered as "New" (US) or "Novel" (EU): where innovative ingredients have little history of use, and safety for human consumption may need to be demonstrated in order to obtain regulatory approval of use. Besides, statements of specific health benefits on food products are subject to scientific review and regulatory approval. "Prebiotic," "probiotic," and "postbiotic" are implicit references to general, nonspecific benefits of the nutrient or food for overall good health or health-related well-being; however, they still have to be accompanied by a specific authorized health claim in the EU (EMA 2006). Health claims may be present on general foods; however, they are particularly prone to be used on food supplements as those are intrinsically expected to provide a nutritional or physiological effect to their consumer (EMA 2002a).

When indicated for therapeutic use, microbiome products may fall within the medicinal product status. Probiotics and postbiotics are also likely to be regarded as biological products in both the EU and the USA as they are typically issued from microorganisms and of biological nature themselves. Probiotics are the only microbiome products susceptible to fall within the Live Biotherapeutic Products (LBP) scope in the USA. Where the cell constituent of the microbiome product (e.g., bacteria, yeasts) has been substantially manipulated or engineered, requirements for Advanced Therapy Medicinal Products (ATMPs) may be applicable in the EU (EMA 2007; see also ▶ Chap. 96, "Toxicological Aspects in the Regulation of Gene Therapy Medicinal Products").

Fecal Microbiota Transplants (FMT) illustrate the variety of regulatory statuses potentially applicable to microbiome health products. While the approach is rather close to a graft, human stool is usually not considered to be a constituent part of the human body itself, which may conflict with existing Regulations applicable to cells and tissues, defined in the EU and the USA as human materials (EMA 2004; FDA 2019a). Where administered to treat *Clostridium difficile* infection, FMT meets the definition of a biological product as per the FDA definition (FDA 2016a). The status of FMT is not harmonized in the EU community, and no specific framework has been designated in most Member States (MS). The European Commission has advised that FMT does not meet the definitions of "tissues and cells"; however, they are considered substances of human origin (European Commission Directorate General for Health and Food Safety – Competent Authorities on Substances of Human Origin Expert Group (CASoHO E01718) 2019). Some MS regulate FMT under Tissues and Cells standards (2), others as Medicinal Products (non-ATMPs) (5) or as Foods (1) (European Commission – Commission Staff Working Document 2019).

The potential regulatory scope of application for microbiome health products is outlined in Fig. 1.

The lay language used for microbiome products is not suitable with respect to regulatory considerations. Prebiotics and probiotics may both be developed as foods (e.g., functional foods or food supplements with health claims; specialized nutrition) as well as therapeutic products (e.g., drugs, biologics, tissues, and cells), and probiotics may in addition to these be developed as LBPs. Additional product characteristics must be taken into account to determine the applicable regulatory status and the associated requirements for development and marketing of a microbiome product in the EU and USA (e.g., intended use, presentation form, nature, and origin of components).

Global Regulatory Framework in Place

A single guidance for all microbiome-related products would be impractical and potentially lead to generic recommendations with little added value. The different regulatory approaches for each status are reflected by the structure of the Authorities framework, either divided in multiple expertise centers (e.g., FDA Center for Biologics Evaluation and Research, Center for Food Safety and Applied Nutrition), or even distinct agencies (e.g., European Medicines Agency, European Food Safety Authorities, National Regulatory and Scientific Agencies).

On the global scale, only limited specific guidance is available to sponsors with regard to microbiome products development. The FDA has published two guidance documents dedicated to microbiome therapeutic products, namely: 1) live-biotherapeutic products (FDA 2016b), and 2) fecal microbiota transplantation (FDA 2016a). Additionally, an interagency Microbiome Working Group has been established in the USA, comprising members from various NIH Institutes and Centers and the FDA, to support discussion of microbiome-related topics and research.

Fig. 1 Regulatory statuses potentially applicable to microbiome health products in the EU and the USA, in the food and therapeutic arenas. (**a**) Prebiotics and postbiotics may be food or drugs, depending in particular on their intended use. In the food arena, they may fall within any status where they may be considered as acceptable food components. They may also fall within many medicinal statuses, including biologicals. Prebiotics and postbiotics are not expected to be considered LBPs; (**b**) Probiotics may fall within all of these same statuses and are additionally particularly prone to be considered LBPs in the USA

No comparable harmonized regulation has currently been established in the EU. Some national agencies have taken the initiative to provide national recommendations for products such as FMT, leading to regulatory discrepancies within the

European Community. Fecal microbiota is regarded as a medicinal product or as human material (ANSM 2016; Hoge Gezondheidraad 2015; Peri et al. 2019). FMT is being regarded as a therapeutic intervention in some MS, explicitly not being subject to the national regulations applicable to drugs, medical devices, or transplantations (Kump et al. 2014). Hospital exemptions also allow custom-made ATMPs to be used in a hospital setting and under the responsibility of the medical practitioner, for a specific patient in individual EU member state, without the need for a marketing authorization (EMA 2018). While the need for an increased cooperation on classification of FMT has been acknowledged by many competent authorities (CAs) (EMA 2004), no common initiative has been initiated to date by the EU Authorities. Inversely, the current trends in the evolution of the regulatory framework for microbiome products are being settled on a national level, e.g., a law project is being evaluated in France which would regulate activities with regards to stool collection (Assemblée Nationale 2019).

The current regulatory framework dedicated to microbiome products is limited. Although the FDA and some EU national authorities have initiated drafting a framework for microbiome products, it remains restricted to therapeutic probiotics (LBPs, FMT). In the existing standards, the recommendations in terms of toxicological assessment remain high level. Prebiotics and postbiotics have not yet been subject to any standardization process. International dialogue between regulatory authorities still needs to be instigated to harmonize the regulation of microbiome products, as outlined by the OECD report on Microbiome Science (OECD 2017). Multiple associations and groups have emerged from research and industry to support and promote the development of microbiome products, such as the Intestinal Microbiome-based Medicines European Task Group (IMM-ETG), the International Probiotics Association, or the Microbiome Therapeutics Innovation Group, demonstrating the need for a globally harmonized standardization.

Stakes and Hurdles of the Toxicological Assessment of Microbiome Products

Use of microbiome-based products could be distinguished in two main therapeutic approaches: 1) displacing pathogenic microorganisms and restoring symbiosis, and 2) delivering genetically modified bacteria to secrete and locally deliver active substance.

Before initiating any clinical studies, developers of microbiome-based products need to demonstrate product safety for their intended use. Yet, as products are typically composed of commensal or historically known living microorganisms, developers might mistakenly consider these microorganisms as safe by definition while neglecting that the formulation, route of administration, and dose can considerably influence the microorganism's activity with a potentially negative impact on the human organism. As example, the presence of the facultative anaerobic *E. coli* in the human gastrointestinal tract is estimated at 10^{21} cells (Conway and Cohen 2015; Kittana et al. 2018). *E. coli* presents a large diversity of strains playing key roles in

the intestine and also encompasses pathogenic variants able to provoke severe intestinal or extraintestinal diseases in humans (Leimbach et al. 2013). Likewise, *Streptococcus pneumoniae* (*S. pneumoniae*) present in the commensal state in the human upper respiratory tract also has capability of becoming pathogenic after reaching normally sterile locations (e.g., lung, bloodstream) (Weiser 2010). Thus, a correct selection of the strain, the dose, and a complete control of the microbiome-based product are essential to ensure safety.

Developers might also consider that the historic use of microorganisms in the food field could be adequate to demonstrate the safety of the microbiome-based product. According to the European Food Safety Authority (EFSA), the Qualified Presumption of Safety (QPS) is based on reasonable evidence. Criteria for such qualification include taxonomic identification, established safety, and absence of pathogenic properties. If those criteria are met, the group of microorganisms evaluated is granted QPS status. A full safety assessment is not required for microorganism belonging to a group with QPS status (European Food Safety Authority (EFSA) 2020). In comparison, in the USA, substances that are reasonably expected to become components of foods are subject to premarket approval by the FDA, unless the substance is generally recognized as safe (GRAS). GRAS classification can be based on scientific procedures or on experience based on common use in food (FDA 2019b). The food regulatory landscape does not require monitoring nor vigilance of potential adverse events associated to food products. Developer may nevertheless find of interest to generate clinical data to support the health claims of their food products. However, clinical data generated for food products cannot be necessarily leveraged to support the use of their ingredients as medicinal products. In the therapeutic field, drug monitoring consists of various measurements, including pharmacokinetics, in order to assess if dosage needs adjustments (Kang and Lee 2009). This quantification is directly correlated to safety assessment, hence crucial even if the product cannot be directly measured in the blood. Thus, relying on experience from the food field to assess microorganism safety may not be sufficient to determine potential toxicological effects.

Hence, customized and reliable nonclinical models (in vitro, ex vivo, and in vivo (Table 1)) have been developed and/or optimized over the past decades by both sponsors and contract research organizations (CROs) to conduct pharmacological and toxicological assessment of microbiome-based products.

Models for Toxicology Assessment

Given the need to support translation to the human situation, establishing models to reflect the complexity of the human microbiota, composed of approximately 100 trillion cells, is a challenge (Fritz et al. 2013). A nonexhaustive outline of currently available in vitro, ex vivo, and in vivo models is presented in Table 1.

In addition to classic animal models, nonclinical approaches include germ-free (GF, also referred as axenic animals) and gnotobiotic (from Greek "known" "life," i.e., in this case, with defined microbiota) animals. Although the first GF animals

Table 1 Overview of available nonclinical study models for microbiome research field

Type	Models	Clinical indication (s)	Target organs/ systems	Advantages	Drawbacks	References
In vitro models	**SHIME** (Simulator of the Human Intestinal Microbial Ecosystem)	Chronic inflammatory diseases (e.g., inflammatory bowel disease, metabolic syndrome)	Mimics the human gut with five compartments simulating the stomach, small intestine, ascending, transverse, and descending colons	Able to simulate the entire intestinal tract. Highly reproducible and stable. Control the colon microbiome ecological aspects. Multiple adaptative models available (e.g., M-SHIME, TWIN-SHIME, TRIPLE-SHIME) allowing for example parallel testing (control vs. test). Long in vitro studies (from one to several weeks). Possibility to do metabolic studies.	The possibility to study the host-microbe interactions is lacking. Long adaptive period (14–21 days). Lack of dialysis.	Fritz et al. (2013), Pham and Mohajeri (2018), Van de Wiele et al. (2015), Chassaing et al. (2017)
	PolyFermS model – Fermentation model	Ecological and metabolic studies	Replicates the adult proximal colon	Simultaneous testing in comparison to a control reactor. Highly reproducible. Studies the impact of environmental parameters, dietary compounds, drugs, and microbes. Stable and reproducible.	The possibility to study the host-microbe interactions is lacking. Lack of dialysis.	Zihler Berner et al. (2013)
	In vitro coculture models	Host-microbe interaction studies	System allowing the separation of microbial and human cell cultures by semipermeable membranes	Avoid the interruption of cocultures of human and microbiota cells).	The possibility to study the host-microbe interactions is lacking. Risk of cross contamination. Strictly anaerobic microbiota setting up not possible.	Fritz et al. (2013), Pham and Mohajeri (2018), Castaneda et al. (2019), Pearce et al. (2018)

(continued)

Table 1 (continued)

Type	Models	Clinical indication (s)	Target organs/ systems	Advantages	Drawbacks	References
	TNO gastro-intestinal model (TIM) TIM1/TIM2 models	Digestive processes studies	In vitro gastrointestinal tract system Stomach and small intestine (TIM1). Large intestine (TIM2).	Mimics the stimulations occurring in the stomach (including, e.g., water absorption, peristaltic mixing, fermentation processes). Allow metabolic studies. Replicate drug's bioavailability.	The possibility to study the host-microbe interactions is lacking. The luminal and mucosal microbiota cannot be separated.	Fritz et al. (2013), Pham and Mohajeri (2018), Keller et al. (2017), Pearce et al. (2018)
	Human cells lines (e.g., Caco-2 cell line, HT-29 cell line, IEC-6 and IEC-18 cell lines)	Gastrointestinal process, host-microbe interactions	Can be used in combination with in vitro fermentation models	Can be used for preliminary investigations. Rebuild a human bacterial microenvironment.	Not representative of GIT system by themselves – Need to be associated with an in vitro system.	Fritz et al. (2013), Pham and Mohajeri (2018)
Ex vivo models	**Explant cultures**	Inflammatory disease (e.g., ulcerative colitis, Crohn's disease)	Colon or rectum (e.g., organotypic slices from human colon biopsies)	Representative of human architecture and cells populations. Reflect in vivo situations by maintaining immune system components, epithelial organization, and metabolic activity.	Mucus layer damaged or removed during the surgical procedure. Artificial treatments (e.g., antibiotics) needed. Not possible to modulate cell populations. In vivo situations can be simulated for a short period of time only.	Fritz et al. (2013), Schwerdtfeger et al. (2019)

In vivo models	**Nematode worm *Caenorhabditis elegans***	Discovery and study of fundamental microbiome biological mechanisms, immune responses	Host-microbiome interactions studies	Well-defined model. Possible to engineer GF and gnotobiotic subjects. Subjects optically transparent, allowing real-time visualization or fluorescence approaches.	Studies on GF subjects may be restricted to young larvae. Microbivores.	Douglas (2019)
	Drosophila	Discovery and study of fundamental microbiome biological mechanisms	Extensively used to study the immune system and the gut microbiota and host-microbiome interactions	Well-defined model. Possible to engineer GF and gnotobiotic subjects. Can be engineered and maintained over multiple generations. Can be engineered by showing a specific microbiota. Similarities with the human gut (e.g., gut divided into three sections). Relatively simple microbiota with only 5–30 taxa. Highly manageable, small size of animals, easy to store, and allowing large sample sizes (statistical precision).	Cannot be humanized by the colonization of human gut microbes (e.g., the Drosophila hindgut (third section of the gut, equivalent to the human colon) is not a fermentation chamber). Different diet as human. Studies on GF may be restricted to young larvae. Microbivores.	Douglas (2018), Kostic et al. (2013), Selkrig et al. (2018), Douglas (2019)

(continued)

Table 1 (continued)

Type	Models	Clinical indication (s)	Target organs/ systems	Advantages	Drawbacks	References
	Zebrafish	Microbiome ecological studies (e.g., impact of arsenic) and metabolic disorders (e.g., type 2 diabetes mellitus)	Mainly used to study host-microbe interactions	Well-defined model. Possible to study host-microbiome interactions -GF and gnotobiotic subjects). Intestinal segmentation and architecture comparable with the mammalian intestine (e.g., similar gene expression and transcriptional regulation in intestinal epithelial cells, the presence of a common protective mucus layer (with mucin) in both zebrafish and mammalian GIT or the presence of an enteric nervous system (ENS). Subjects optically transparent, allowing real-time visualization or fluorescence approaches. Short generation time. Easy to store (measured 3–4 cm long). Fully sequenced genome. Biologically reproducible–Interesting for interindividual variation studies.	Mammalian GIT is divided into five distinct part (stomach, duodenum, jejunum, ileum, and colon), whereas the zebrafish is divided into three segments (anterior intestinal bulb, middle intestine, and posterior intestine). Zebrafish presents a less complex intestinal architecture as human. Lives in an aquatic environment. Difficult and costly to rear GF zebrafish into adults (90–100 days necessary to reach adulthood). Omnivores but different diet to humans (aquatic insects, crustaceans, and plant materials).	Flores et al. (2019), Fritz et al. (2013), Stephens et al. (2016), Dahan et al. (2018), Okazaki et al. (2019), Douglas (2019), Rawls et al. (2007)

	Mouse	Host-microbe interaction studies: Host nutrition and disease development	Allows to explore various interactions between the gut microbiota and, among others, mammalian physiological aspects such as: Brain development, obesity, malnutrition, and hepatic function	One of the most used in vivo animal models to study host-microbiota interactions. Anatomical, histological, and physiological similarities between mouse and human intestines. Genetic similarity between inbred strains allows the reproducibility of the experiments. Well-defined models. Genetic similarities between mice and human: 99% of mouse genes shared with human. Gnotobiotic[a] mice can be colonized with human gut microbiota and maintained over several generations with the defined microbiota. Mice have the same organs as human. Small size – low sheltering cost.	Different organ size scale size (e.g., the large intestine reaches up to 14 cm in mice and 105 cm in human). Small intestine of 33 cm in mice and 700 cm in human. Large intestine up to 14 cm in mice and 105 cm in human. Total transit can last 14–16 h in human vs. 6–7 h in mice. Mucus growth rate higher in the human colon than in the mice colon (240 +/− 60 μm/h vs. 100 +/− 60 μm/h). Different metabolic rate. Different diet habits. Mice are hindgut fermenters with grain-based diets, humans are omnivores. Mice have a nonglandular forestomach, higher stomach pH in mice than in human (pH 2.7–4.1 vs. > pH 1). Mouse skin differs vs. human skin. Not fully relevant for skin, oral cavity, upper respiratory tract, and genitourinary tract studies.	Franklin and Ericsson (2017), Fritz et al. (2013), Hugenholtz and de Vos (2018), Kibe et al. (2005), Kostic et al. (2013), Lavin et al. (2018), Nagpal et al. (2018), Le Roy et al. (2019), Rosshart et al. (2019), Goodman et al. (2011)

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Table 1 (continued)

Type	Models	Clinical indication (s)	Target organs/ systems	Advantages	Drawbacks	References
					Human microbiota vs. mice microbiota differ in quantities. Approximately 80 microbial gut genera in common. Faecalibacterium, Succinivibrio and Dialister were not found in some laboratory mice. Different immune system – Difficult to predict humans' complex immune responses. Exclusive herbivores. Higher pH in the stomach. Laboratory mice may present different microbiota.	
	Wilding mice Laboratory mice embryos (C57BL/ 6) transferred into wild mice – Next generation	Immunological and microbiome-related disorders	Mice model counterbalancing the drawbacks of laboratory mice (e.g., variable microbiota)	Animals microbiota remain stable throughout generations. Animals remain robust against microbial, antibiotic, and dietary challenges. Animals have the same modifiable genetics of C57BL/ 6 mice. Good translation model into humans (e.g., immunological responses). Reproducible.	See above (e.g., organ size, mucus growth rate, metabolic rate, diet, stomach pH).	Rosshart et al. (2019)

	Rats	Host-microbe interaction studies: Host nutrition and disease development	Gut microbiota	Rat microbiota better reflects the human microbiota than mice. Physiological and anatomic similarities with human GIT (organ, tissue, and cells). Large size facilitating the evaluation of metabolic parameters (e.g., blood pressure). Genome fully sequenced. Can better support human fecal microbiota than mice.	Exclusive herbivores. Higher pH in the stomach. Higher mucus growth rate in humans than in rats. Faster digestion as in humans. Low-fiber digestion capacity.	Flemer et al. (2017), Fritz et al. (2013), Lleal et al. (2019), Marques et al. (2015), Nagpal et al. (2018), Heinritz et al. (2013)
	Rabbits	Sinusitis	Sinus microbiota	Similar sinonasal epithelium immunologic features. Sinusitis model well-defined.	Surgery and bacteria inoculation can enhance changes in mucocilial transport.	Cho et al. (2018)
	Dogs	Gastrointestinal disorders (e.g., acute hemorrhagic / nonhemorrhagic diarrhea, idiopathic inflammatory bowel disease) Skin	Skin microbiota and gastrointestinal disorders	Skin conditions: Can develop atopic dermatitis similarly to humans. Possible to quantitate liver glucose levels. High fecundity. Diarrhea often occurs after weaning in research puppy colonies.	High mortality rates (high sensitivity to some disease models). Need extra care being domestic animals.	Kleinert et al. (2018), Burton et al. (2016), Rodrigues Hoffmann et al. (2014), Suchodolski et al. (2012), Coelho et al. (2018)

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Table 1 (continued)

Type	Models	Clinical indication (s)	Target organs/ systems	Advantages	Drawbacks	References
	Pigs	Gastrointestinal diseases (e.g., stress-induced intestinal dysfunction, intestinal ischemia / reperfusion injury, colorectal cancer, short-bowel syndrome, weaning diarrhea, necrotizing enterocolitis, obesity) Esophageal metaplasia and neoplasia Metabolic disorders (e.g., diabetes mellitus, obesity)	Particularly relevant to study esophageal disease, intestinal ischemia / reperfusion injury or even psychological stress-induced intestinal dysfunction	Anatomic, physiological, genomic (fully sequenced, with 60% of similarities with human), proteomic, immunologic, metabolic, and nutritional similarities with human. Esophagus (with the esophageal submucosal glands), stomach, structure of the small intestine, intestinal length and weight, and epithelial cell population are very close between the two species. Omnivore. Able to perform fermentation in their colon, which have sacculations and longitudinal muscular bands (like human colon). Close microbiota within their small and large intestine (mainly constituted of Firmicutes and Bacteroides, as humans). Comparable clinical signs and pathophysiological signs as human when submitted to stress. Well-developed central and peripheral nervous system enabling to integrate complex	Pigs have no appendix. Pigs cecum is much more developed and larger than the human cecum. Pigs have a different spinal arrangement in their colon. Slow reproduction rate (4 months gestation) compared to rodents.	Gonzalez et al. (2015), Ziegler et al. (2016), Crespo-Piazuelo et al. (2018), Heinritz et al. (2013), Zhang et al. (2013)

				stress signaling processes. High fecundity. Experimentally tractable animal model. Possible translation of the human microbiota into pigs.		
	Non-human Primates (NHP)	Gastrointestinal disorders Metabolic disorders Pathogenesis and sexual infection transmissions (e.g., vaginal microbiota)	Ideal to study the vaginal and rectal microbiota	Behavioral, physiological, and genetic resemblances with humans. Widely used model in the medical research field – Well-known. Similar gut, skin, and genital microbiota when compared with other species (e.g., mice and rats).	Differences in the gut, vaginal, and saliva microbiota Higher quantities of Proteobacteria in rhesus macaques and baboons vs. higher quantities of Bacteroides and Firmicutes in human gut microbiota. Lower quantities of Lactobacillus in NHP than in human vaginal microbiota. Human and macaques saliva microbiota share three prevalent taxa: Streptococcus, Haemophilus, and Veillonella.	Chen et al. (2018), Nagpal et al. (2018), Kennedy et al. (2018), Kleinert et al. (2018), Nugeyre et al. (2019)

ENS Enteric Nervous System, *GF* germ-free, *GIT* Gastrointestinal Tract, *NHP* nonhuman primate(s)

[a]Gnotobiotic – Germ-free animals can become gnotobiotic models when colonized with specific and defined germs (Fritz et al. 2013; Pham and Mohajeri 2018)

were established in the late 1940s, their actual potential to conduct microbiome studies was recently implemented (Al-Asmakh and Zadjali 2015; Kennedy et al. 2018; Fritz et al. 2013; Pham and Mohajeri 2018). Gnotobiotic animal models evolved alongside the increasing understanding of the microbiota. They enable the conduct of studies to determine the functional impact of a defined microbiota on host physiology (Dewhirst et al. 1999; Marcobal et al. 2015; Martín et al. 2016; Basic and Bleich 2019). Despite obvious limitations, GF and gnotobiotic models have been broadly used to characterize patient – microbiota interactions in toxicology investigations and to investigate drug impact on host microbiota. Therefore, evolving models would need to focus on investigating microbiome-based products and their impact on both host health and host microbiota.

Toxicological Assessment Strategy

Demonstrating safety and efficacy remains a prerequisite for all new medicinal product development, as well as determining an optimal dose and dosing regimen using nonclinical studies. The goals of nonclinical development as regard to microbiome-based products are the same, and so the strategy for demonstrating a positive outcome should be specifically tailored to the microbiome medicinal product. Using commensal bacteria is not an evidence per se of their safety, yet. The stakes are even higher for LBPs based on genetically modified bacteria or for therapies using phages to selectively re-program patient's targeted bacteria strain. The bacteria could be modified ex vivo through classic genetic engineering, or by the use of a new process involving clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated Cas proteins (Hamilton et al. 2019; Munck et al. 2020; Ramachandran and Bikard 2019). These genetically modified organisms (GMO) are usually designed to either selectively modify or eliminate members of the patient microbiota. Phage therapies implement bacteriophage (i.e., bacteria-specific viruses) natural competence to selectively target and kill bacteria and are aimed to be an alternative approach to the use of antibiotics (Paule et al. 2018; Divya Ganeshan and Hosseinidoust 2019; Vitetta et al. 2018). The sponsor needs to ensure the lack of toxicological effects of LPBs toward the patient both at local and systemic levels, and toward patient's microbiota to ensure a selective and controlled action. Of note, sponsors will also have to investigate risks associated to the propagation of the GMOs in the environment.

Toxicology assessment will have to focus both on the potential adverse effects of the microorganisms themselves and of the molecules derived from these microorganisms. Bacterial molecules (e.g., proteins or polysaccharides, lysates), surface molecules (e.g., surface markers, capsid proteins), or secreted molecules (e.g., proteins including but not limited to toxins and polysaccharides) could in fact have a direct or indirect impact on patient health. Therefore, the nonclinical strategy could then be compared to the strategy used to demonstrate safety and efficacy of cell and gene therapies. The sponsor will need to demonstrate safety of their microbiome-

based product and investigate pharmacokinetic (biodistribution, colonization, and elimination/shedding) and toxicology effects on the patient and his/her microbiota. Therefore, the choice of using one or two species (rodent and nonrodent) will also rely on which toxicological effect needs to be investigated. The guideline ICH M3R (2) will apply for products composed of molecules of bacteria or phage origin (secreted molecules or lysates) (ICH M3(R2) 2009). In the case of LBP, toxicology assessment strategy could be compared to what is done for cell and gene therapy products (see ▶ Chap. 96, "Toxicological Aspects in the Regulation of Gene Therapy Medicinal Products"). Given the large variety of microbiome-based products and targets, the toxicological assessment will have to be considered on a case-by-case basis. For instance, a sponsor developing bacteria-derived proteins will not investigate their toxicological potential for developing a LBP; nor would products aiming to eradicate bacteria be assessed the same way as products aiming to restore microbiota symbiosis.

Distribution, Biodistribution, and Colonization

Even if not part of the toxicological assessment per se, distribution (of secreted molecules), biodistribution, and colonization/engraftment should be investigated as often correlated to potential toxicological effects. This is even more important when investigating LBPs which are usually aimed for long lasting effects to restore population balance in the patient microbiota. Therefore, the sponsor should ensure that these microorganisms reach the target site/organ appropriately and does not migrate to undesired sites. Their persistence should also be evaluated as these bacteria should accordingly colonize the targeted area but not over-proliferate within the host or in the environment.

Immunogenicity/Immunotoxicity

All microbiome-based products may have a potential toward a drastic immunologic impact by different paths: they may elicit a host immune response toward bacterial molecules; they may also affect host immune system by altering host reactions; and/or they may affect the immune system by jeopardizing the patient homeostasis.

Gut microbiota are known to play a key role in the maintenance of homeostasis and health (Buret et al. 2019; Crespo-Piazuelo et al. 2018; Kostic et al. 2013; Martín et al. 2016; Vitetta et al. 2018). Same ascertainments have been made with other epithelium-associated microbiota such as skin (Johnson et al. 2018; Muszer et al. 2015; Nakamizo et al. 2015), oral (Solbiati and Frias-Lopez 2018), and vaginal (Chen et al. 2018; Nugeyre et al. 2019) microbiota. Clinical data suggest that probiotics and prebiotics may have immunomodulating capabilities by altering the intestinal microbial populations and tempering the activity of some resident bacteria (termed pathobionts) which have the potential to cause disease.

Genotoxicity and Tumorigenicity

Genotoxicity and tumorigenicity potential assessment are expected for pharmaceutical products, even more so when the product itself has been genetically modified. Sponsors need to investigate the capability of their product to modify and/or damage the patient cell DNA by various mechanisms. However, developers of microbiome-based products are confronted with the lack of transposition of the standard regulatory guidelines for pharmaceuticals (ICH S2(R1) 2011) or for biotechnology-derived pharmaceuticals (ICH S6(R1) 2011). The requirement of genotoxicity and tumorigenicity studies may be waived if sponsors can demonstrate that tumor development, if any, cannot be correlated to microbiome products and/or associated secreted molecules during any of the pharmacology or toxicology studies. Hence for ATMPs, genotoxicity and tumorigenicity will be assessed on a case-by-case basis. However, carcinogenesis can be triggered by specific bacterial pathogens. For example, Helicobacter pylori (*H. pylori*) can develop gastric cancer in infected patients (Li and Perez Perez 2018; Tibbs et al. 2019; Francescone et al. 2014). *H. pylori* and other bacteria can also induce chronic inflammation and an associated increase in reactive oxygen species (ROS)-mediated genotoxicity (Tibbs et al. 2019; Francescone et al. 2014). Therefore, the investigation of both genotoxicity and tumorigenicity will be probably requested by the regulatory agencies, and sponsors are therefore expected to propose a nonclinical plan based on their product specific features and as appropriate for the intended clinical use (FDA 2016b). Methods to assess genotoxicity could eventually be transposed and adapted to microbiome-based products, with appropriate controls to be included to validate such assays (Federici et al. 2017; González Pereyra et al. 2014; Endres et al. 2009).

Reprotoxicity

As for cell and gene therapies, reprotoxicity studies should be determined using a risk-based approach. Reprotoxicity risks could be identified from the intrinsic nature of the product, from preliminary findings on reproductive organ analysis, from the pharmacology and toxicology studies, and from the route of administration (e.g., vagina, urinary tract) (ICH M3(R2) 2009; ICH S5(R2) 2005). Reprotoxicity studies may then be deemed necessary. Of note, the guideline ICH S5 is currently under revision (ICH S5(R3)) since 2015, but it still does not take into consideration evaluation specific to microbiome-based medicinal products neither to cellular nor gene therapies, nor tissue-engineered products (ICH S5(R3) 2020).

The appropriate time-course should be established on a case-by-case basis and the assessment revisited throughout the development of the product. Endres et al. chose, for example, to investigate their LBP through combined overall toxicology assessment and reprotoxicity assessment (Endres et al. 2009; Endres et al. 2011). The requirement for reproductive toxicity studies may be waived if sponsors can demonstrate that the microbiome products and/or associated secreted molecules do not distribute in the gonads.

The relevance of such studies should be discussed early in the development with the agencies, and a follow-up discussion may eventually be needed depending on the nonclinical results collected prior the clinical phases.

New Nonclinical / Clinical Approach: In Silico Modeling and Simulation

While new nonclinical models have been established to better understand the microbiome and develop microbiome-based medicinal products, in silico (meaning done with a computer) modeling and simulation (M&S) methods (Rousseau et al. 2019) have demonstrated to complement nonclinical and clinical studies. Historically, M&S was limited to early stage research analyses, to support medical device development, or to physiologically based pharmacokinetic modeling (PBPK) applied to small molecule development. However, in the past few years, developers as well as the agencies have started to acknowledge the potential of M&S contributing to product development by providing a new type of evidence: the digital evidence. Like microbiome science, M&S use is an emerging field for which the regulatory framework is currently being built. They will among other things support the development of applications to manipulate microbiome (OECD 2017). Nevertheless, researchers have already identified the potential use of M&S applied to the microbiome field (Moorthy and Eberl 2017; Borenstein 2012; Bauer and Thiele 2018; Greenhalgh et al. 2019; Michelini et al. 2018). Moorthy et al. proposed an in silico platform to investigate host microbiome, its interactions, and its role on the host health (Moorthy and Eberl 2017). Other research teams also developed M&S strategies to explore microbiome impact on immune system and even identify personalized microbiota (Bauer and Thiele 2018; Michelini et al. 2018).

In summary, conducting a toxicology assessment of microbiome-based products will most likely require sponsors to combine multiple nonclinical models, best determined based on each model's limitations.

Bench to Bedside Translation

Translation challenges between nonclinical models and the human situation are acknowledged (OECD 2017). However, with the rise of microbiome-based medicinal products, new nonclinical models have been developed, as described in Table 1. Sponsors can therefore identify appropriate nonclinical model(s) from a panel of models to investigate their microbiome-based medicinal product. As mentioned in section "Stakes and Hurdles of the Toxicological Assessment of Microbiome Products," germ-free and gnotobiotic models offer broad possibilities to investigate various microbiome populations (Kennedy et al. 2018; Kittana et al. 2018; Lavin et al. 2018; Vlasova et al. 2018).

Microbiome-based products aim to modify/interact with the patient's microbiota and, therefore, are not designed to act toward the patient's cells/tissues.

Their action is indirect on the patient and contributes to modifying or eliminating target members of the patient's microbiome community. This paradox epitomizes the difficulty in classifying these products into a regulatory framework. Since they may be genetically modified microorganisms or perhaps, they might lead to genetic modification of the patient's microbiota, should they therefore be considered as gene therapies? By the same token, when products consist of selected bacteria populations aimed to colonize patients' tissues, would those products thus be considered cell therapies?

To optimize the bench to bedside translation, sponsors should consider an integrated approach with regards to the Chemistry, Manufacturing, Controls (CMC), and nonclinical and clinical development as this will directly impact nonclinical outcomes and their relevance toward the expected human outcomes.

Conclusions

Microbiome-based products, as all health-care products, have to demonstrate that their quality, safety, and efficacy fit their intended use. Due to their large variety and mode of action, the nonclinical to clinical translation for microbiome-based products provides specific development challenges not typically encountered with other types of regulated healthcare products. This fact is also reflected in the difficulty for regulators and developers to classify them. Given the products' intrinsic features, nonclinical studies, and especially the toxicology assessment, should be tailored following a case-by-case approach. This can be supported by a vast spectrum of microbiome-adapted nonclinical models already well-developed and constantly improving.

Despite an increasing interest for such products, the regulatory framework is currently evolving alongside knowledge, with regulatory agencies and developers working in conjunction to build this regulatory framework (see also ▶ Chaps. 39, "Biomolecules Versus Smaller Chemicals in Toxicology: ICH, EU, and US Recommendations," and ▶ 96, "Toxicological Aspects in the Regulation of Gene Therapy Medicinal Products"). Therefore, it is of utmost interest for sponsors to reach out to regulatory agencies early on and during development to evaluate together the fitness and robustness of any proposed nonclinical and clinical development plans.

Cross-References

- ▶ Biomolecules Versus Smaller Chemicals in Toxicology: ICH, EU, and US Recommendations
- ▶ Toxicological Aspects in the Regulation of Gene Therapy Medicinal Products

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Toxicological Aspects in the Regulation of Gene Therapy Medicinal Products

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Cécile F. Rousseau, Emmanuelle Sabbah-Petrover, Deborah Revaud, Emmanuelle M. Voisin, Manfred Ruthsatz, and Carlo Chiavaroli

Contents

C. F. Rousseau · E. Sabbah-Petrover · D. Revaud · E. M. Voisin
Voisin Consulting Life Sciences, Boulogne, Paris, France
e-mail: rousseau@voisinconsulting.com; sabbah@voisinconsulting.com; revaud@voisinconsulting.com; voisin@voisinconsulting.com; vcparis@voisinconsulting.com

M. Ruthsatz
Nutrition+HealthCARE, Vevey, Switzerland

C. Chiavaroli (✉)
Voisin Consulting Life Sciences, Lausanne, Switzerland
e-mail: chiavaroli@voisinconsulting.com

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Abstract

The safety of Gene Therapy Medicinal Product (GTMPs) is of paramount importance. Therefore, the European Medicines Agency (EMA), the US Food and Drug Administration (FDA), and other agencies in the world have issued regulatory guidelines/guidance. Nevertheless, as the field is evolving so fast and safer vectors being developed, a continuous adaptation of the regulation is needed, and not surprisingly, some nonclinical requirements deemed necessary only a few years ago (such as assessing the tumorigenicity of GTMPs in vivo), may appear less relevant today. As any GTMP has its own specificities, a "one-size fits all" toxicology development program cannot be routinely imposed by competent authorities. Rather, a scientifically driven and tailored approach, considering the choice of the delivery vector, the gene to be corrected, and the expected benefit/risk for the targeted indication is recommended for each GTMP. It is advised to present and discuss the development strategy early to regulatory agencies to agree upon the most appropriate design of the nonclinical studies to be conducted.

This chapter informs sponsors on the putative toxicological requirements for their specific GTMP. A review of the evolving regulatory toxicology landscape is provided, for both the EU and USA. To capitalize from the lessons learned from the past to illustrate potential toxicological requirements for new therapies, a benchmark of toxicology studies performed with the eight GTMPs approved by December 31, 2019, in the EU and/or USA is presented.

A hypothesis on the future regulation for the toxicological assessment of GTMPs is provided in the conclusion.

Keywords

Gene therapy · Toxicology · Vectors · Tailored approach · Benefit/risk

Introduction

For those old enough to remember the limits of our phones and computers commercialized in the seventies, a parallel can be made to illustrate what is happening in the gene therapy arena: these technologies evolved remarkably during the last decades and both will undoubtedly continue to progress at increasing speed. Therefore, the safety for humans of gene therapies is mandatory, and regulatory agencies need to

adapt to continuous changes. Importantly, the standard regulatory guidelines applicable to, for example, "Pharmaceuticals" (i.e., ICH M3(R2) 2009) or "Biopharmaceuticals" (ICH S6(R1) 2011) cannot be fully transposed to gene therapies. For example, one major difference between GTMPs and conventional small molecule pharmaceuticals or other biological products is that once a GTMP has been administered it can be difficult to discontinue the effect of the expressed corrected protein or enzyme, especially when using integrative vectors.

Historically, it was only in 1990 that the first gene therapy trial approved by the US Federal Drug Administration (FDA) was launched when a four-year-old girl, named Ashanthi DeSilva, underwent a 12-day treatment for a rare genetic disease known as severe combined immunodeficiency (SCID) (Gruntman and Flotte 2018). This patient lacked the enzyme adenosine deaminase (ADA), a key enzyme for immune cells, and was successfully treated. Initial successes were however tempered by some abrupt but necessary slow-downs, for example, in 1999 after the death of an 18-year-old boy suffering from ornithine transcarbamylase deficiency (the patient experienced a severe immune reaction to the vector, (Sibbald 2001)), or when insertional oncogenesis was found in four young SCID-X1 patients and resulted in T cell leukemia after gene transfer of IL2Rγ via a Moloney murine leukemia virus (MoLV)-based gamma-retroviral vector into Hematopoietic Stem Cells (HSC) (Hacein-Bey-Abina et al. 2008; Couzin and Kaiser 2005).

These serious warnings allowed the emergence of safer vectors and improved techniques. Promising tools such as Clustered Regularly Interspaced Short Palindromic Repeats/Caspase 9 (CRISPR/Cas9)-based therapies are emerging, which not simply allow to incorporate a corrected gene into human cells, but also to replace a mutated "diseased" gene with its "healthy" counterpart. Thus, in 2019, the FDA has received more than 800 active Investigational New Drug (IND) applications involving GTMPs. At least eight gene therapies are currently approved between EU and USA (and four other in China, Philippines, and Russia), and it is extrapolated that 40 gene therapies should be approved by 2022 (Elverum and Whitman 2019).

Main Gene Therapies and Associated Technologies

A series of definitions is provided in section "Definitions and Associated Tests and Assays" of this chapter for further guidance.

Main Vectors for Gene Therapies (With or Without Ex Vivo Modified Cells)

Gene therapies can be achieved using genome nonintegrative or integrative approaches, with viral or nonviral vectors. The focus of this chapter is mainly on viral vectors. These vectors play a dual role in the context of gene therapies: either directly acting on the host genome or acting as delivery tools for gene editing systems (such as Clustered Regularly Interspaced Palindromic Repeats (CRISPR)/Cas9).

Gene therapies always involve the insertion of a correct copy of (at least) a defective gene, or a modification of a gene by correcting a mutation, or the inactivation of a gene. GTMPs in general aim to restore or inactivate (a) gene function(s) as cause of pathogenic symptoms. Gene therapies *sensus stricto* include (1) therapies in which the treatment consists of direct administration of only the vector containing the gene(s) of interest (i.e., without cells), and (2) gene-modified cell therapies (i.e., with cells, either from autologous or allogenic origin) in which target cells are modified ex vivo) using the vector containing the gene(s) of interest and then administered to the patient.

In any case, the transgenic expression should be sufficient in terms of level and duration to definitively correct the targeted disease. An ideal vector should be nonimmunogenic and safe and deliver the desired gene to a specific cell type or tissue. The benefit/risk balance of both nonintegrative or integrative (in the human genome) approaches and the associated gene transfer vectors (and technologies) need to be considered according to the planned clinical indication and the patients' population, taking into account previous knowledge and the safety assessments already reported in animals and humans with related vectors (and technologies).

Ideal vectors should be able to transport large therapeutic genes and achieve high transduction efficiency (Goswami et al. 2019). They should provide long-term and stable expression, as well as target specific cells and/organs, avoid random insertion of the therapeutic gene into the host genome, and modify mitotic as well as postmitotic cells. It should not be immunogenic nor pathogenic, nor cause inflammation and should possess the ability to be manufactured on a large scale.

The main advanced vectors for gene therapy can be grouped in the major classes shown in Table 1: plasmids and viral vectors derived from genetically modified natural viruses. The two main viral vector families currently used for gene therapy in clinical studies are recombinant adeno-associated viruses (rAAV*)* and retroviral/lentiviral vectors.

Plasmids and Virus-Derived Vectors Overview

Plasmids

Plasmids are closed, circular DNA strands and can be inserted directly into targeted tissues as nacked-DNA, where they remain within the nucleus, thus limiting the risk of insertional mutagenesis. Since 1990s, the use of plasmids as nonviral (bacterial) vectors for gene delivery has been recognized (Wolff et al. 1990). A diversity of physical and chemical methods including for instance needles, electroporation, gene guns, ultrasounds, photoporation, magnetofection, hydroporation, mechanical massage, lipid, calcium phosphate, silica, and gold nanoparticles can be used to deliver the genetic material to target cells. Nonviral vectors such as plasmids also allow to select almost unlimited transgene size.

Because of their improved safety profile compared to viral vectors, plasmids have been widely tested in human clinical trials (Hardee et al. 2017). As they are poorly immunogenic, they can be administered via a dose-repeated regimen.

Table 1 Overview of main vectors, associated features, and toxicological concerns

Gene therapy vectors	Structure/integration	Number of clinical trials	Pros	Cons
Plasmids	Circular DNA Nonintegrative, remain episomal in the nucleus	260[a]	Less immunogenic that viral vectors Repeat administrations possible Can accept larger transgenes than rAAV and LV	Plasmids can be diluted during cell proliferation Low delivery efficiency requiring methods that may impact DNA integrity Risks of genome integration
rAAV	Single stranded DNA + capsid Considered as nonintegrative, the majority of AAV DNA remain episomal in the nucleus	202 AAV[a] + 49 rAAV[a]	Generally recognized as nonpathogenic and less immunogenic than other viral vectors (e.g., lentiviral vectors) Robust stability (i.e., shelf-life, little or no loss of activity with wide temperature and pH changes) **Tissue specificity (which may vary among species) can be achieved by selecting appropriate capsid sequence(s)** **AAV-1**: preferred tropism for neuronal, cardiac, and skeletal muscle cells in various animal models Used in 12 clinical trials (up to Feb 2020) and **for approved gene therapy Glybera** **AAV-2:** broad specificity Used in 63 clinical trials (up to Feb 2020) and **for approved gene therapy Luxturna** **AAV-5:** preferred tropism for airway, retinal receptors, pigmented epithelium, and CNSUsed in 9 clinical trials (up to Feb 2020) **AAV-6:** Preferred tropism for skeletal muscle and lung **AAV-8**: preferred tropism for liver, heart, skeletal muscle, and pancreas Used in 20 clinical trials (up to Feb 2020) **AAV-9**: Preferred tropism for liver, heart and skeletal muscle,	Limited DNA size that can be inserted (generally 4.7 kb) Diluted during cell proliferation Preferred tropism of AAV may be species-dependent **Potential safety issues:** AAVs targeting liver may lead to hepatotoxicity Rare events of vector integration with a preference for sites close to active genes, ribosomal DNA, and CpG sequences Immunogenicity could be detected (preexisting immunogenicity to former AVV infection) AAV correlated with potential ocular toxicity (**mainly AAV-2 and AAV-8**)

(continued)

Table 1 (continued)

Gene therapy vectors	Structure/integration	Number of clinical trials	Pros	Cons
			spinal cord, and brain Used in 9 clinical trials (up to Feb 2020)	
Lentiviral Vectors	Double strand RNA + capsid + envelope Integrative at nontargeted site in the genome	198 lentiviral vector[a] + 87 labeled as retroviral vector[a]	High efficiency of transduction on broad cell-types. Can accept transgenes above 4.7 kb (rAAV limit), however the transduction efficacy decreases with size **1st Generation:** The vesicular stomatitis virus G protein (VSV-G) improves the stability and broadens the cellular tropism of the viral particles produced **2nd Generation:** HIV accessory proteins not essential to the production of the LV particle have been removed. The lentiviral genes are split across at least three plasmids. The risk of generating RCL is lower **3rd Generation (and Beyond):** Currently, the third-generation lentiviral vector system offers the best safety profile in terms of RCL generation because this vector requires only three HIV-1 genes (out of 9) (gag, pol, and rev) for production. The plasmid encoding the gene of interest contains lentiviral LTR sequences that have been altered to be self-inactivating (SIN) to prevent recombination. Effective delivery of genetic material and maintenance of long-term stable expression in target cells, delivery of larger amounts of genetic material than other methods, do not cause an inflammatory response in the recipient **Used in approved gene therapies: Kymriah and Zynteglo**	Potential risk of insertional mutagenesis (tumorigenesis), immunogenicity towards envelope protein limits repeat-dose administration **1st Generation:** potential risk for the generation of Replication Competent Lentiviruses (RCL) especially if HIV positive human cell are used at the clinical stage. Potential risk of tumorigenesis **2nd Generation:** Still extra-care is needed when working with proto-oncogenes or with human samples that have not been tested for HIV. **3rd Generation (and Beyond):** Recognized safe for clinical development. However, as "Risk zero" level is impossible to achieve, decisions to treat patients should be based on reasonable benefit/risk considerations

[a]Numbers of clinical trials identified on www.clinicaltrials.gov consulted February14, 2020

Recombinant AAV Vectors

It is important to highlight that single-stranded DNA AAV (adeno-associated viral vectors) should not be confused with adenoviruses (double-stranded DNA), which resulted in the death of a patient who suffered from ornithine transcarbamylase deficiency, a rare X-linked genetic disease leading to metabolic disorder, during a gene therapy clinical trial in 1999 (Sibbald 2001). Indeed, despite the similarity in name, AAVs are fundamentally different from adenoviruses.

The amino acid sequence of the viral capsid proteins determines the serotype of AAV. Thirteen different AAV serotypes have been identified, which differ in their tropism and hence the target organ, tissue, or cell type (Srivastava 2016). This feature can be used to developed organ/tissue/cell type-specific gene therapy to control the delivery of the therapy after administration, limiting the risk of toxicity for an off-target organ/tissue/cell, as summarized in Table 1. To achieve such a broad tropism, AAVs require the expression of heparin sulfate proteoglycan, avb5 integrin, a5b1 integrin, fibroblast growth factor receptor 1, platelet-derived growth factor receptor, hepatocyte growth factor receptor, epidermal growth factor receptor, laminin receptor, and/or sialic acid moieties on the surface of target cells for efficient transduction and delivery (Goswami et al. 2019).

Retroviral/Lentiviral Vectors

Retroviral vectors developed for gene therapy were initially derived from Moloney Murine Leukemia Virus (MoMLV). Viral particles were produced based on a two plasmids system: (1) the packaging plasmid containing the viral structural genes (*gag*, *pol*, t*at*, r*ev*, and *env*), (2) the plasmid containing the target gene of interest and the sequence necessary to encapsulate the target gene into viral particles (Psi signal).

Retroviral vectors derive from RNA genome viruses and integrate permanently into the human genome via a reverse transcription mechanism. A delivered gene persists overtime and propagates through cell replication. Lentiviral vectors derived from Human Immunodeficiency Virus (HIV) belong to the family of retroviruses. A lentiviral vector can target broader cell types and therefore is considered as interesting tools for the development of gene therapy.

Efficacy of retroviral vectors in clinic has been demonstrated for ex vivo gene therapy in hematological diseases (Nienhuis 2013). Indeed, lentiviral vectors are considered as a tool of choice to develop gene therapy to target hematopoietic stem cells (HSC) (Morgan et al. 2017; White et al. 2017).

Despite leukemia being reported in patients treated with a retroviral vector for X-linked SCID (Hacein-Bey-Abina et al. 2008) and Wiskott-Aldrich syndromes (Braun et al. 2014), in 2016 the EMA granted its approval to Strimvelis®, a retrovirus expressing the Adenosine Deaminase (ADA) protein, for the treatment of ADA deficiency-associated SCID disease (Aiuti et al. 2017; Stirnadel-Farrant et al. 2018). The toxicology approach conducted with Strimvelis® is summarized in Table 2.

CRIPSR/Cas9 Technology

Recently new perspectives emerged with the development of genome editing tools such as CRISPR/Cas9 system. Genome editing offers a much more precise technology for altering genes. CRISPR/Cas9 is a genome editing technology developed to quickly, easily, and accurately correct gene mutation and to turn on or off gene function (Hsu et al. 2014; Estêvão et al. 2018). While CRISPR/Cas9 has mainly been studied in animals for the development of human disease models, it presents many potential human applications (Mokbel and Mokbel 2017), offering new perspective of gene therapy development to treat genetic disorders caused by single mutation, by deleting or inserting gene sequences.

CRISPR/Cas9 system is a prokaryotic enzymatic machinery that is reconstituted in mammalian cells using two essential components: (1) Cas9 (CRISPR-associated protein 9), an endonuclease that cuts DNA in a sequence-specific manner using (2) a single-guide (sg) RNA with a sequence that matches the target gene (Hsu et al. 2014). The resulting DNA double strand break (DSB) is then repaired by the DNA repair machinery. The genomic region is disrupted, and gene corrected for gain or loss of function. Plasmids containing the sequence of sgRNA and of *cas9* gene are usually used to deliver CRISPR/Cas9 tool within cells, although Cas9 protein can be delivered directly.

The current human clinical trials with CRISPR/Cas9 gene therapy are all at early stage, in three main areas: cancers, blood disorders and eye disease. The first, still ongoing US-based clinical trial uses ex vivo CRISPR/Cas9 technology (NCT03399448, (Baylis and McLeod 2017)) for the engineering of immunotherapy (e.g., CAR T-cells therapy). Cells from patients are transfected in vitro with plasmids encoding for sg RNA and Cas9 protein (or Cas9 protein directly delivered). Ex vivo genome editing enables to control that only targeted patient cells are in contact with the genome editing tool prior to being administered intravenously.

Toxicological Aspects in the Regulation of GTMPs

Both the European Union (EU) and the United States (US) agencies have adopted guidance specific to gene therapy medicinal products. GTMPs include a broad variety of products including but not limited to plasmid DNA, viral and nonviral vectors, genetically modified viruses or cells that are developed for treatment or prevention of a variety of human diseases. EMA and FDA guidance has therefore evolved as scientific knowledge and experience have grown alongside of new therapies being developed. Indeed, product-specific and scientifically based deviations from established classical guidelines (initially implemented for small molecules) are increasingly needed to address the nonclinical development of innovative gene therapies.

In this rapidly evolving arena, the European Medicines Agency (EMA) has lately updated a guidance released in 1999 on the quality, nonclinical, and clinical aspects of gene therapy medicinal products for the purpose of Market Authorization

Application (MAA) (EMA 2019a). The FDA has recently published a draft guidance on interpreting the sameness of gene therapies under the orphan drug regulations (FDA 2020a), and several guidance documents specific to gene therapies in regards to Chemistry, Manufacturing, and Control (CMC) aspects relevant to gene therapies (FDA 2020b, f) and for various clinical indications such as hemophilia (FDA 2020c), retinal disorders (FDA 2020e), or "rare diseases" (FDA 2020d).

Though some differences exist between the EU and US guidelines, the approach is quite similar in the sense that a risk-based approach should be followed for the elaboration of the nonclinical and clinical development plan of GTMPs (EMA 2013a; FDA 2006). The extent of the toxicity studies as well as their design should be based on the vector selected (integrative; nonintegrative and discussion around the possibility of random integration; replication competent virus formation and insertional mutagenesis); preexisting data with similar vector backbone; use of cells modified ex vivo; route of administration; as well as dose/dosing regimen and number of administrations (e.g., discussion around the safety margin; repeat dose or not); indication targeted and the expected mode of action.

General Toxicology

As a consequence of the human specificity of GTMPs, selecting nonclinical appropriate model(s) responding similarly to the human system is a true challenge and of utmost importance. In this context, both the EMA and FDA concur that the use of nonrelevant test systems and animal models (healthy or diseased, wild type or immunocompromised) should be avoided as this could lead to the wrong appraisal of the expected benefit/risk for patients. Since clinical trials (including first-in-human studies) with GTMPs are conducted in patients and not in healthy volunteers, the use, when available, of an animal model of disease for the safety evaluation of the therapy appears appropriate and can be recommended, as diseased animals may be closer to the patients expected to receive the gene therapy than wild-type animals. When possible, sponsors are even encouraged, in observance of the *3Rs* principle (Reduce/Refine/Replace), to optimize in vivo studies allowing extraction of multiple data (pharmacodynamics/biodistribution/toxicity) from a reasonable number of animals. In this context it is worth to mention that, in contrast to what is usually performed for the development of small molecules, it is not necessary to perform the toxicity studies in two different species (one rodent and one nonrodent). Usually rodents, that is, mice (or rat, more rarely), often immunocompromised NSG (NOD scid gamma) mice lacking mature T cells, B cells, and natural killer (NK) cells are selected to avoid rejection of human cells. In the facts it is rare to collect data for GTMPs in dogs or monkeys (one noticeable exception is Luxturna®, approved in 2018 and in 2017 in the EU and USA, respectively).

Although toxicity studies must be complaint with Good Laboratory Practices (GLP) and standards according to 21 CFR Part 58 and the WHO Handbook for Good Laboratory Practice, the regulators recognize that it may be difficult in some circumstances to perform all assessments under GLP compliance (e.g., disease

animal models are usually not available in GLP facilities) (World Health Organization 2009). When deviations are unavoidable, they should be properly justified in the dossier, including the impact on the data generated and conclusions. The EMA has released a short memo on GLP and Advanced Therapeutic Medicinal Products (ATMP) development (EMA 2017).

For nonclinical studies and more importantly for toxicity assessment, the investigational product used should be representative of the one that will be used in clinic (though not necessarily of Good Manufacturing Practices (GMP) grade); comparability/bridging studies are usually strongly advised to help the evaluation of its "representativeness" (FDA 2013). The product used in the toxicity study should have the same "potency" (biological activity) than the one to be used in clinic and should bare the same quality attributes as well as impurity profile. A specific paragraph on that matter is included in both the FDA guidance on nonclinical assessment of investigational cellular and GTMPs and on the EMA guideline on ATMPs, for the purpose of marketing application and investigational ATMPs. The EMA has also published in 2011 a reflection paper on modifications of GTMPs during development to share its views on potential impact of any changes in the development including nonclinical plan (EMA 2011). If a competent authority has any doubt regarding the comparability of the product used in the toxicity studies and the clinical batch, it may ask the developer to repeat the toxicity studies with a more representative batch. Hence, in case of doubt, it is strongly advised to seek advice from regulatory agencies before performing the GLP-compliant study.

The toxicity evaluation, as part of these studies, should assess local and systemic potential toxicity of the gene therapy and should be tailored to the intended clinical product. The use of the same animal model in both toxicology investigations and pharmacokinetics (biodistribution) studies is recommended, in particular when vector-related toxicity signals are observed (EMA 2018a). It is essential to emphasize that, for such product, the usual (expected) biological effect(s) and clinical benefit(s) are most likely on the long-term (lifelong). Hence, the duration of the general toxicity studies for gene therapies may be longer than for other investigational medicinal products, considering that usually only a single administration of the gene therapy is performed. However, when more than one administration is envisaged in humans, the number and timing of administrations in animals should at least be equal to and be representative of the intended clinical regimen. Also, it is acknowledged that usually the Maximum Feasible Dose (MFD) should be assessed during the toxicological evaluation, unless appropriately justified.

Biodistribution

Classical Absorption-Distribution-Metabolism-Elimination (ADME) studies are not appropriate for GTMPs; however, the assessment of the biodistribution and potential shedding of the vector are expected. Biodistribution is not considered as part of the toxicology program of a gene therapy per se but is often linked to it. Biodistribution studies are most of the time mandatory for GTMPs, prior to the First-in-Human

administration, especially in the case of a novel backbone, change in the route of administration or significant change in the formulation as well as change in the dose/dose regimen (FDA 2013). The route of administration as well as the dose (including an appropriate safety margin that should be discussed with the Competent Authorities) and dose regimen should mimic as closely as possible the proposed clinical use. As stated in the Guideline on the quality, nonclinical, and clinical aspects of gene therapy medicinal (EMA 2018a), in addition to the proposed clinical route, it is sometimes useful to evaluate the maximum exposure via a systemic route of administration (e.g., intravenous in the case of an intramuscular administration).

Importantly for the future, ICH has released in November 2019 a concept paper for a new guidance, S12: Nonclinical Biodistribution Considerations for GTMPs. The objectives of this future guidance are to provide harmonization in the regions covered by the ICH on the definition of biodistribution; the need for and timing of the conduct of such studies as well as information on the methodologies and the data needed to justify the selection of most relevant species for pharmacology and safety studies; finally, discussion on the interpretation of the biodistribution data for clinical study design will be included. This guidance is expected to be released by 2023.

In addition to the regulatory texts, the readers are invited to consult a very informative review, presenting an EU perspective (Silva Lima and Videira 2018). To decrease the number of animals during the development, it may be useful and relevant to combine toxicity studies with biodistribution and shedding in accordance to the *3R*'s principle. This would also ease the assessment of the causality of any toxicity to the product.

Biodistribution is usually assessed by administering the GTMPs either in healthy animals, or in immunocompromised mice (e.g., NSG mice), or in an animal model of the disease (depending on model availability; and potential "impact" of the condition on the biodistribution). The animals (males and females) are sacrificed at different time points; organs and tissues sampled and prepared for qPCR specific to the vector or more generally to nucleic acid hybridization techniques (NAT). It is usually recommended to have at least two time-points (in NSG mice, the latest time point is frequently measured 4 months after the administration of the GTMP) demonstrating clearance or plateau of the distribution to be able to conclude.

Vector shedding (in the body fluids) is an important feature to assess during the development, especially in the case of a new vector construct (where it is strongly recommended to generate data prior to the first administration to human). Results of shedding studies will indeed have consequences on the clinical development to define the containment level in the clinical settings and the procedures for preparation, administration then waste management as well as the associated safety measures. Shedding can be evaluated along biodistribution (i.e., distribution of the gene therapy within the body) or toxicity studies, to avoid using specific animals for that matter in agreement with the 3R's principle. As for other evaluations, relevance of shedding studies and when these should be performed should be assessed on a case-by-case basis (*c.f.* EMA guidelines on (investigational) ATMPs (EMA 2018a, 2019a).

For shedding studies, body fluids and/or excreta are collected at specific timepoints then typically quantitative (RT-)PCR or droplet digital PCR (ddPCR) being more sensitive can be performed to quantify the amount of vector present.

As regards the genomic distribution, it is crucial to assess the potential risks associated with the use of a defined vector, especially in the case of a new vector construct. To do so, integration site analysis is usually performed following in vivo administration of the gene therapy and sampling and preparation of DNA samples that are then digested and run through linear amplification mediated (LAM)-PCR.

Finally, biodistribution results in the gonads are important factors in assessing probability of germline transmission, as elaborated in the EMA guidance on non-clinical testing for inadvertent germline transmission of gene transfer vectors (EMA 2006).

Insertional Capability

Relevance of performing insertional mutagenesis evaluation should be assessed on a case-by-case basis, depending on vector type; dose to be administered (the more vector copies, the higher the probability of insertional mutagenesis); cells or organs targeted; and the transgene expressed. Of note, previous use and knowledge of the vector should also be taken into account.

The most widely used test for assessing insertional mutagenesis is the in vitro immortalization assay (IVIM), based on in vitro culture of murine bone marrow lineage depleted cells (Modlich et al. 2006). In this assay, the replating frequency of transduced cells is measured and evaluated per vector copy number.

Due to genome integration and potential insertional mutagenesis, patients treated with lentiviral vectors are followed for any emergent signs of clonal cell proliferation. In addition, recombination between lentiviral vectors and wild-type lentivirus is a safety concern due to a potential risk for the generation of Replication Competent Lentiviruses (RCL), especially if HIV positive human cells are used at the clinical stage. One assay to assess the risk consists in infecting permissive lines with the vector followed by serial passaging and then PCR of specific regions. "Ideally, the capacity of tests to detect one replication competent lentivirus in a vector dose should be proven." The FDA has released a guidance on the testing of replication-competent retroviruses that provides detailed information, including on the methods, amount of supernatant or product to be tested, and sensitivity of the assay (FDA 2020f).

Recombinant adeno-associated viruses (rAAV) developed for gene therapy are deleted for sequences encoding viral proteins necessary for their integration in the human genome, contrary to wild-type associated adenovirus (AAV). The majority of rAAV remain episomal after transduction (Schnepp et al. 2003, 2016). Therefore, they are considered to present a good safety profile with regards to potential risk

of insertional mutagenesis and then associated genotoxicity and tumorigenicity. However, recent nonclinical data gathered in murine model suggest that rAAV could also result in insertional mutagenesis leading to genotoxicity (Chandler et al. 2017).

Genotoxicity and Tumorigenicity

Genotoxicity remains a crucial aspect of the toxicity program for gene therapies. Such nonstandard tailored evaluations are very different from what is classically required as per (ICH S2(R1) 2011) for small molecules. GTMPs can indeed have oncogenic activity. Genotoxicity studies can be performed in silico, in vitro, ex vivo, and/or in vivo (EMA 2008). Oncogenic potential of GTMPs should in principle be evaluated first *in silico* (e.g., presence of oncogene protein sequences, or mode of action of the GTMP in the genome).

The EMA Guideline on the quality, nonclinical, and clinical aspects of GTMPs (EMA 2018a) indicates that "standard life-time rodent carcinogenicity studies are usually not required in the nonclinical development" but rather suggests that an evaluation on their relevance should be based on several parameters including the mode of action of the GTMP and previous results such as insertional mutagenesis studies, hormonal perturbation or immune suppression and other relevant red flags. This is also indicated in the draft guideline on investigational ATMPs: "The need for additional toxicity studies for instance genotoxicity, tumorigenicity, reproductive and developmental toxicity, and immunotoxicity studies should be determined on a case by case basis taking into consideration the risks related to the nature and characteristics of the particular class of ATMP and the intended clinical use" (in Guideline on quality, nonclinical, and clinical requirements for investigational advanced therapy medicinal products in clinical trials (EMA 2019a)). When deemed necessary, tumorigenicity is usually assessed in vivo, by administration of the investigational drug in immune-deficient animals and then monitoring of the potential appearance of mass.

In summary, if an oncogenic potential has been identified in silico, then tumorigenicity should be evaluated in appropriate in vitro and/or in vivo models, for example, by analyzing first the proliferative capacity of the cells, response to apoptosis stimuli and genomic modification) (EMA 2008). The extent of studies for nonintegrative vectors can be certainly less than for integrative vectors. However, some data or thorough discussion will still be required to justify at least a low probability of insertional mutagenesis and tumorigenicity events. Only when deemed necessary following positive in vitro positive data, then developers should test tumorigenicity in vivo, in, for example, immunodeficient NSG mice for GTMPs consisting of ex vivo transduced cells to avoid immune rejection, to measure the occurrence of leukemia/lymphoma following administration of the cells. If rare events are detected, then additional analyses are needed to rule out that they were caused by the gene therapy.

Off-Target Toxicological Effects: Case of Genome Editing CRISPR/Cas9 System

In addition to potential immunogenicity of the delivery system and Cas9 protein, the other main safety issue when using the CRISPR/Cas9 gene-editing tool is the possibility to observe off-target effects such as genome editing in off-target site, which may cause mutagenesis in normal genes or mis-repair of DNA double-strand breaks (DSB) leading to genetic instability and formation of chromosome aberrations. Hence, the use of CRISPR/Cas9 for the development of gene therapy in biomedical and clinical application raises safety concerns associated with potential tumor development (Albertson et al. 2003).

To minimize off target effects of CRISPR/Cas9, respective optimizations are ongoing (Zhang et al. 2015) and focus on (1) Specific design of single guide (sg) RNA sequence to limit the tolerance of base pair mismatches and the recognition of nontargeted region of the genome, (2) control of the concentration of Cas9 protein and sgRNA delivered to control the level of activity and anti-Cas9 immune reaction, (3) development of mutant versions of Cas9 protein to render the nuclease function even more specific.

Immunogenicity (Immunotoxicity)

Preexisting exposure to Cas9 protein and to viral vectors such as AAV may result in immunogenicity after a single administration of the GTMP to patients. Therefore, new in vivo nonviral delivery systems are under development to decrease immune reaction and ensure efficient transport to the target cells (Shim et al. 2017).

Whatever the type of vector selected for a GTMP, an "immuno-toxicological" assessment will be required, by checking, for example, inflammatory responses, immunogenicity, and/or autoimmunity. Immunogenicity studies for gene therapies include also as appropriate humoral and cellular immune response endpoints to measure the potential innate and adaptative immune responses to the vector itself and/or the transgene. This is particularly important in the case of repeat administration to evaluate the potential immunogenicity.

Immunogenicity is a major aspect to be evaluated. Immunogenicity could be directly related to the composition of the vector or its packaging (e.g., capsid protein, cell proteins). In the case of rAAV vectors, host reaction towards the capsid proteins needs to be evaluated. Such reaction often translates in humans into a transient elevation of the liver transaminase level, indicating a liver damage. This transient change in liver physiologic parameters is due to the host immune reaction towards the AAV capsid protein and can be managed with a steroid treatment (Colella et al. 2018). As the transduction rate with AAV constructs appears lower than obtained with lentiviral vectors, AAV doses to achieve similar levels of gene expression should be higher, and this could potentially increase the risk of immunogenicity. Of note, retroviral vectors could as well be associated to autoimmune reactions (EMA 2016).

Reprotoxicity

Reprotoxicity should be determined by a risk-based approach. Both the US and EU guidance indicate that if deemed necessary, reprotoxicity results should be provided before confirmatory/Phase 3 studies in which usually large populations are studied. The appropriate timepoint though should be established on a case-by-case basis and the assessment revisited throughout the development of the product. For instance, if the vector and gene products does not distribute in the gonads, the requirement for reproductive toxicity studies may be waived. In the same perspective, if no biodistribution is seen in the gonads, gene transfer studies in descendant animals may not be needed).

The EMA guideline on investigational ATMP states that "*if effects on reproductive function and/or development are anticipated relevant reproductive and developmental toxicity studies should be conducted before exposing larger patient populations*" (EMA 2019a). Though some flexibility is acknowledged in the EMA guideline on gene therapies for MAA, it is at the same time clearly stated that "*Embryo-fetal and perinatal toxicity studies and germline transmission studies should be provided, unless otherwise duly justified* on the basis of the type of product concerned." Very interestingly, the draft guideline ICH S5(R3) on Detection of Toxicity to Reproduction for Human Pharmaceuticals (currently at stage 2 of the ICH review process) indicates it does not apply to Gene Therapies (ICH S5(R3) 2017).

It is worth mentioning that the risks associated with for instance lentiviral exposures have been described at length (Schlimgen et al. 2016). This author does not cite "reprotoxicity" as a potential risk. In contrast, White et al. report that the standard battery of reproductive function tests as per (ICH S5(R2) 2005) is generally not required unless otherwise highlighted by specific features of a proposed therapy (e.g., if the gene therapy is specifically targeted toward the reproductive organs) (White et al. 2017).

However, developers should also consider the risks associated for the reproductive system with the potential overexpression and specificities of their transgene(s).

Translational Risks from Bench to Clinic

Over the past two decades, gene therapy *sensus stricto* and gene-modified cell therapy product development has been increasing exponentially. The alliance for Regenerative Medicine reported 362 clinical trials for each of these approaches in its 2018 Annual Report (Alliance for Regenerative Medicine 2018), most of them being in Phase 1 or Phase 2 of their clinical development. Figures went up by Q3 2019 to 370 and 418, for gene therapy and gene-modified cell therapy, respectively (Alliance for Regenerative Medicine 2019). Nevertheless, translational research faces challenges such as how to demonstrate the reliability of nonclinical models towards what could be expected in First-in-Human (FIH), assessing the dose-efficacy relationship as well as risk/benefit ratio for patients.

Some of the risks associated to GTMPs are inherent to the products themselves. Nonclinical assessment should be therefore conducted on a risk-based approach while the nonclinical development plan will have to be tailored to the product to be evaluated. Ultimately, developers will have to demonstrate to the Regulatory Agencies that sufficient nonclinical data has been collected to support moving forward into a FIH study, and minimize the risks for patients. One common misconception is that one nonclinical model will have to be THE relevant model to address all safety concerns. Such a unique model is typically not available, as the nonclinical development must be tailored and adapted to the GTMP, combining several nonclinical models. Using in vitro, ex vivo, in vivo models, and even in silico modeling and simulation enhances the chance of ensuring successful translation to clinical studies. Lastly, the proper choice of negative (and positive) controls is of course crucial to assess the relevance of the data obtained.

All agencies agree on a key crucial point: sponsors have to demonstrate the relevance of the models they chose to investigate their product(s) and define any limitations of these models. Based on our experience, establishing the scientific validity of nonclinical data supporting the transition to a First-in-Human clinical trial is clearly more challenging for GTMPs when compared to traditional small molecules. To increase chances of translation to clinical setting, nonclinical development should be performed in alignment with the clinical intended use for the target population, while considering the specificities of the CMC of the GTMP.

Limitations of nonclinical models are acknowledged, and they may lead to clinical study failures (Couzin and Kaiser 2005). Sponsors need to demonstrate to the regulatory agencies the relevance and limitations of the selected model(s) and not overestimate the translation of the collected data for the human situation.

Finally, one of the primary risks associated with GTMPs lies in the effect and impact of overexpression or absence of expression of the protein/enzyme of interest. In such cases, understanding the distribution of the vector/gene within the whole body, and not only in the targeted organ(s) or tissue(s), becomes crucial.

Benchmark with Approved Gene Therapies

From public information available in the Summary Basis for Approvals (SBAs) and European Public Assessment Reports (EPARs), we identified five GTMPs approved before the end of 2019 in the USA (Imlygic®, Kymriah®, Luxturna®, Yescarta®, and Zolgensma®), and seven in the EU (Imlygic®, Strimvelis®, Kymriah®, Luxturna®, Yescarta®, Zyntenglo®, and Zolgensma®, and in addition Glybera® was approved in the EU in 2012 but has been withdrawn from the market at the marketing holder's request) (Paul-Ehrlich-Institut (PEI) 2020). A tabulated summary of toxicology investigations performed on various GTMPs is presented in Table 2.

In addition, four other gene therapies (not presented in Table 2) are authorized in other regions of the World (Goswami et al. 2019): Gendicine® (Head and Neck Cancer, since 2003 in China), Oncorine® (Nasopharyngeal Carconoma, since 2005

Table 2 Approved gene therapies in the EU and/or USA

Approved gene therapies[a]	Approval year	Sponsor/applicant	Indication	Vector and enzyme expressed	Toxicology and biodistribution studies conducted
Glybera® (EMA (2012b) only)	2012 (*withdrawn from the market*)	uniQure biopharma B.V.	Treatment to reverse lipoprotein lipase deficiency. This therapy has been voluntary withdrawn from the market by the applicant	Replication-deficient adeno-associated virus type-1 (AAV1) vector expressing the human LPL gene variant LPLS447X	Five GLP-compliant **biodistribution** studies were conducted (**mice, rabbits, cats**). Shedding was addressed in clinical trials **GLP toxicity studies** (IM, single dose, up to 180 days) were performed in **mice**. Different doses were tested Studies on **insertional mutagenesis** done Female mice were treated 4 weeks prior to mating to **asses transmission of vector DNA to fetuses** **Reproductive toxicity** was studied in pregnant mice **Local tolerance** as part of toxicology studies
Imlygic® (EMA 2015; FDA)	2015	Amgen Europe B.V.	Treatment of melanoma that is regionally or distantly metastatic	An attenuated nonintegrating Herpes simplex virus type-1 (HSV-1) virus coding granulocyte macrophage colony-stimulating factor (GM-CSF)	**Biodistribution, viral shedding** (urine, feces, lachrymal glands, nasal mucosa, salivary glands), and **replication studies** conducted in **mice** **Toxicology studies** (up to 5 administrations) up to 12 weeks

(continued)

Table 2 (continued)

Approved gene therapies[a]	Approval year	Sponsor/applicant	Indication	Vector and enzyme expressed	Toxicology and biodistribution studies conducted
					in **mice** and an **embryo-fetal development study in mice** (GLP). **Additional single dose studies** were also conducted in **rats and dogs** An **embryo-fetal toxicity** study conducted in the **mouse**, as well as a **placenta transfer study**
Strimvelis® (EMA (2016) only)	2016	GlaxoSmithKline Trading Services Ltd.	Treatment of ADA-SCID patients	A gamma-retroviral MoMLV vector (GSK3336223) encoding adenosine deaminase (ADA) cDNA sequence	No nonclinical studies were performed to assess vector shedding after GSK2696273 administration Selected **general toxicity** endpoints were assessed in the definitive **Biodistribution** study in mice. **NSG mice** deficient in mature T/B/NK cells ($n = 10$/sex/group) were preconditioned with busulfan prior to dosing transduced cells (single IV dose of $CD34^+$ cells derived from human UCB transduced with either the GSK3336223 vector (GSK3235757A) or mock-transduced The EMA agreed that the tumorigenicity risk assessment

					for GSK2696273 could be based on clinical data and literature on similar vectors
Kymriah® (EMA 2018b; FDA)	2018	Novartis Europharm Limited	Treatment of N-cell acute lymphoblastic leukemia (ALL) and diffuse large B-cell lymphoma (DLBCL)	Replication-defective, third-generation self-inactivating (SIN) LV vector encoding a chimeric antigen receptor (CAR) directed against hCD19	One **biodistribution study** (up to D56) done in NOG mice. As tisagenlecleucel is a one-shot patient specific product, which is not appropriate to administer to immune competent animals, no standard single-dose or repeat-dose toxicity studies have been conducted, however an **in vivo safety assessment** was made in a **murine leukemia xenograft model** Non-GLP LV **insertion site analysis** (LISA) done on selected human ALL and HV samples (and also via shearing-extension primer tag selection and ligation-mediated PCR) The **toxicity of some impurities and excipients** were also assessed A **tissue cross-reactivity study** was done, as well as an immunohistochemistry, in situ hybridization and RTPCR analysis on **human and cynomolgus monkey CNS tissues**

(continued)

Table 2 (continued)

Approved gene therapies[a]	Approval year	Sponsor/applicant	Indication	Vector and enzyme expressed	Toxicology and biodistribution studies conducted
Luxturna® (EMA 2018c; FDA)	2018	Spark Therapeutics Ireland Ltd.	Treatment of Leber's congenital amaurosis or retinitis pigmentosa inherited retinal dystrophy	An AAV2 capsid with human retinal pigment epithelium 65 kDa protein (hRPE65) gene	**Distribution** studies were included as a component of general toxicity studies **Toxicity and local tolerance** studied in **Briard (RPE65$^{-/-}$) dogs** and in animals with **normal RPE65 status (dogs, monkeys)**. Virus was injected subretinally either once only, or once into each eye, and twice into the same eye (12 days apart)
Yescarta® (EMA 2018d; FDA)	2018	Kite Pharma EU B.V.	Treatment of Non-Hodgkin's lymphomas	Retroviral vector PG13-CD19-H3 is a murine stem cell virus (MSCV)-based vector pseudotyped with the gibbon ape leukemia virus (GaLV) envelope	**Biodistribution and persistence** of the antimurine CD19 CAR T cells evaluated in the syngeneic mouse lymphoma model using flow cytometry analysis No standard single-dose or repeat-dose toxicity studies have been conducted, however **on-target/off-tumor toxicity** of CD19 CAR T cells in the syngeneic mouse lymphoma model, which has been **evaluated during the pharmacology study** in parallel with the anti-lymphoma effect and the persistence of the anti-murine CD19 CAR T cells

Zynteglo® (EMA 2019b)	2019	Bluebird bio B.V.	Treatment of β-thalassaemia	A 3rd generation SIN LVV encoding $\beta^{A\text{-}T87Q}$-globin	**A pivotal combined therapeutic POC, pharmacology, single-dose toxicity and genotoxicity study in the β-thalassemic mice** **PK and biodistribution** of BB305 LVV-transduced human healthy donor HSCs were also evaluated **in NSG mice** **Four pivotal single-dose toxicity studies (including local tolerance) in primary and secondary recipient mice**
Zolgensma® (FDA)	2019	AveXis, Inc.	Treatment of spinal muscular atrophy (SMA)	An AAV9 vector encoding Human SMN gene	The **biodistribution and SMN transgene expression** profile of AVXS-101 were evaluated in **neonatal FVB mice through 12 weeks** **Single-dose toxicology studies (IV) in neonatal FVB mice**

[a]Only as of Dec 31, 2019

in China) and Rexin-G® (Pancreatic Cancer, since 2007 in the Philippines), and Neovasculogen® (Peripheral Artery Diseases, since 2011 in Russia).

The studies conducted reflect the specificity of each of the approved GTMPs. Interestingly, reprotoxicology evaluations were performed only with Glybera® and Imlygic®, the most ancient GTMPs approved, and such investigations were not requested for the most recent GTMPs, suggesting that the regulatory appraisal by the competent authorities is taking into account the nonclinical and clinical knowledge acquired through the years.

Definitions and Associated Tests and Assays

Autologous Versus Allogenic

Autologous cell therapy is a therapeutic intervention that uses the patient cells, which are cultured and expanded outside the body, and reintroduced into the donor. In contrast, allogenic product is usually used to refer to a source material from other human donors.

Biodistribution, Persistence, Clearance

For small molecules, classical absorption, distribution, metabolism, and excretion (ADME) studies are usually performed. These studies are not appropriate for gene therapies, for which one rather assesses the biodistribution (of the cells, vector, and expressed transgene) in relevant organs, as well as the persistence, and clearance.

CRISPR/Cas9

The CRISPR/Cas9 technology has emerged recently as a powerful tool to perform genome editing (as analogy, one can visualize this technique as the "copy/paste" shortcuts for text editing in Word). It originates from the natural immune system of bacteria, destroying infecting viruses. Briefly, the CRISPR sequence is composed of repeat nucleotides sequences and spacers. These "blocks" of sequences originate from viruses that previously infected the host. The second member of the CRISPR/Cas9 binomial is Caspase 9, an enzyme that is usually represented as "scissors" as it cuts specific sequences of DNA. Researchers have now developing improved and safer versions of CRISPR/Cas9.

Gene Therapy

In Europe, the definition of a gene therapy medicinal product is provided in Annex I Part IV of Directive 2001/83/EC (EMA 2012a):

Gene therapy medicinal product means a biological* medicinal product which has the following characteristics:

(a) It contains an active substance which contains or consists of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, replacing, adding, or deleting a genetic sequence.
(b) Its therapeutic, prophylactic, or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence."

*Biological is defined in Annex I Dir 2001/83/EC: "*A biological medicinal product is a product, the active substance of which is a biological substance. A biological substance is a substance that is produced by or extracted from a biological source.*" Consequently, the manufacturing process is crucial in determining the product classification

In the USA, the definition is included in federal register vol 58 N°197 (FDA 1993):

GTMPs are defined as products containing genetic material administered to modify or manipulate the expression of genetic material or to alter the biological properties of living cells.

Gene therapy is a medical intervention based on modification of the genetic material of living cells. Cells may be modified ex vivo for subsequent administration or may be altered in vivo by GTMPs given directly to the subject. The genetic manipulation may be intended to prevent, treat, cure, diagnose, or mitigate disease or injuries in humans.

Importantly, both the EU and US definitions of gene therapies clearly indicate that ex vivo modified cells that are then (re) administered to a patient are considered as gene therapy medicinal products; one example is the CAR-T cells products, now approved on both sides of the Atlantic.

Germline Transmission

Germline transmission (known as "*inadvertent gene transfer*" in the USA) is the vertical transmission of DNA sequence to offspring, something which is still clearly prohibited as per the EU legislation. Usually, factors such as the vector type route of administration, dosing, and importantly biodistribution results in the gonads are important factors in assessing probability of germline transmission.

The EMA guideline on nonclinical testing for inadvertent germline transmission of gene transfer vectors (EMA 2006) refers to this topic.

Insertional Mutagenesis

Insertional mutagenesis is the creation of mutations in the host genome by addition of DNA base pairs. Insertional mutagenesis that may potentially lead to oncogenesis is a recognized safety concern of integrating vector-based gene therapies. However, even

for vectors that do not efficiently integrate, such as adeno-associated vectors (AAV), plasmids, or retroviral vectors modified to avoid integrations, insertions events can be detected (though very scarcely) and the risk should still be discussed and evaluated in some circumstances (refer to the Reflection paper on management of clinical risks deriving from insertional mutagenesis released by the CAT in 2013 (EMA 2013b)).

Integrative Versus Nonintegrative Vectors

Some of the vectors used in gene therapy are integrative (e.g., γ-retroviral vectors and lentiviral ones), whereas others are supposed to remain extra-chromosomal ("episomal") such as AAVs though this feature is debated. While γ-retroviral vectors integrate preferentially at promoters and gene 5′ ends, and clustering near genes controlling cell growth and proliferation has been reported, lentiviruses integrate preferentially in transcription units, avoiding regulatory 5′ regions.

NSG Mice

NOD *scid* gamma mice are preferred immunodeficient animals, lacking lymphoid cells with natural killer cell activity almost inexistent. They however display normal growth and are widely used especially in the context of research on hematopoietic stem cell transplantation and/or to assess the log-term toxicological effects of, for example, genetically modified human cells in another species.

Replication-Competent Virus (RCV)

A replication competent vector is a vector able to replicate. Most (if not all) of the viral vectors used in gene therapy medicinal product manufacturing or as direct administration are designed to be replication incompetent for safety purposes. For such vectors, assessment of replication-competent vectors that might have been generated during the manufacturing process or in vivo (following administration) must be performed.

Tumorigenicity

Tumorigenicity should be distinguished from carcinogenicity as the former focuses on the formation of a tumor (i.e., mass of cells), whereas the latter focuses on the development of cancer; though the authors agree that both terms are often used indistinctly.

Vector Mobilization

Vector mobilization is the potentiation (or "rescue") of the gene therapy by a wild-type virus in vivo that could lead to virus (uncontrolled) spread and potentially RCV.

Optimizations in vector design have significantly decreased the probability of vector mobilization by splitting the required sequences.

Vector Shedding

Shedding is the dissemination of the vector through secretions and or excreta of the patient (or animal) (e.g., saliva, urine, feces).

Conclusion and Perspectives

To date, only 12 different somatic gene therapies against cancer, blindness, immune, and neuronal disorders were approved worldwide, including eight in Europe and the United States. To achieve MAA/BLA, each sponsor had to overcome many specific hurdles applicable to their unique GTMP and to provide a strong nonclinical dossier, with comprehensive proof-of-concept, toxicology (usually in rodent only), biodistribution, shedding, and insertional mutagenesis, and sometimes (actually only for Glybera® and Imlygic®), repro-toxicity data. With the exception of Zolgensma®, nonclinical data were collected in adult animals.

These initial approvals should pave the way for many other MAAs / BLAs, as the possibilities for GTMPs appear endless as virtually every cell in the human body is a potential target for genetic manipulation. As for more established medicinal therapies, it appears possible that in the future the nonclinical studies for GTMPs may be abridged, based on the following hypotheses:

1. Only a restricted number of vectors and techniques with well-known benefit/risk profiles will be authorized for new GTMPs (it is our view that new vectors and techniques will however still need to go through an exhaustive nonclinical assessment before being approved for gene therapies, unless otherwise justified).
2. The scientific community and regulators are collecting large amounts of relevant nonclinical and clinical data which may render some of the nonclinical investigations unnecessary (e.g., demonstration of absence of insertional mutagenesis will not be needed anymore with "approved vectors").
3. Finally, the emergence of in silico virtual assessments should considerably decrease the number of "real *in vivo* studies" to be conducted.

Cross-References

► Biomolecules Versus Smaller Chemicals in Toxicology: ICH, EU, and US Recommendations
► Microbiome Product Toxicology: Regulatory View on Translational Challenges

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Defence of Biocidal Active Substance Dossiers in the Context of Regulatory Toxicology

97

Michael Werner

Contents

Abstract

In this chapter, the expertise gathered in the broad area of toxicology and its implementation into the regulatory context is illustrated using the approval process of biocidal active substances as an example.

A brief overview of the relationship between biocidal active substances and product types is given followed by a description of the biocidal active substance approval process from the regulatory point of view. The different steps as well the timelines to be observed will be explained.

With a focus on the toxicological data requirements for biocidal active substances, the upcoming revisions of Annex II of the Biocidal Products Regulations as required by the mandatory assessment of potential endocrine-disrupting properties will be illustrated. The scientific criteria as well as the challenges encountered for the identification of potential endocrine-disrupting properties will be discussed.

Following thorough assessment of the toxicological data package submitted for active substance approval, the final and most important step in the approval

M. Werner (✉)
Wöllstein, Germany

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process consists of the derivation of references values for systemic and local effects, depending on the effects and nature of the active substance. Consideration is given to the need and the type of the references values usually derived for the purpose of the risk characterization in the intended uses of biocides, i.e., acceptable exposure levels (AELs) and acceptable exposure concentrations (AECs). As one further important pillar within the scope of active substance approval, the need for an exposure assessment with regard to the intended uses of biocidal products containing the active substance and the demonstration of acceptable risks will be addressed.

Keywords

Active substance · AEL/AEC · AOP · Biocides · BPC · CfS · Cumulative risk assessment · dCAR · eCA · Endocrine disruption · EOGRTS · Exclusion criteria · Exposure assessment · Hazard · MoA · MIE · KER · Risk characterization · SoCs · Toxicity · Waiver · WoE

Introduction

The broad area of toxicology and its various disciplines focus primarily on the investigation of the potential detrimental effects of natural and synthetic substances on living systems.

For this purpose, not only standardized studies but also studies with a "tailor-made design," such as mechanistic studies, are performed toward the elucidation of the mode of action (MoA) and with a view to hazard characterization and hazard assessment.

Also *in vitro* studies (e.g., on mutagenicity/genotoxicity) form part of the establishment of a complete toxicological data package which in most of the cases is triggered by regulatory requirements of different (horizontal) legislations.

In this context, it is of particular note that especially "higher tier studies" such as repeated dose, developmental and reproductive toxicity, and carcinogenicity studies are usually performed in experimental animals *in vivo* according to recognized international standards such as the OECD guidelines for the Testing of Chemicals or the EPA OCSPP/OPPTs health effects testing guidelines for pesticides and toxic substances as *in vitro* or *in silico* methods are either not available yet or are not capable of completely and satisfactorily addressing these higher tier endpoints.

An integral part of mode of action (MoA) investigations is the clarification of the adverse outcome pathways (AOPs), comprising the determination of molecular initiating events (MIE) as well as the series of key events and their relationships to each other (KERs), finally leading to an adverse outcome (AO) on an organ and/or tissue level.

Following completion of the toxicological data package, a thorough evaluation of all studies and the effects observed therein is performed including a "weight-of-the evidence (WoE) approach" in this assessment. In addition, the process of hazard

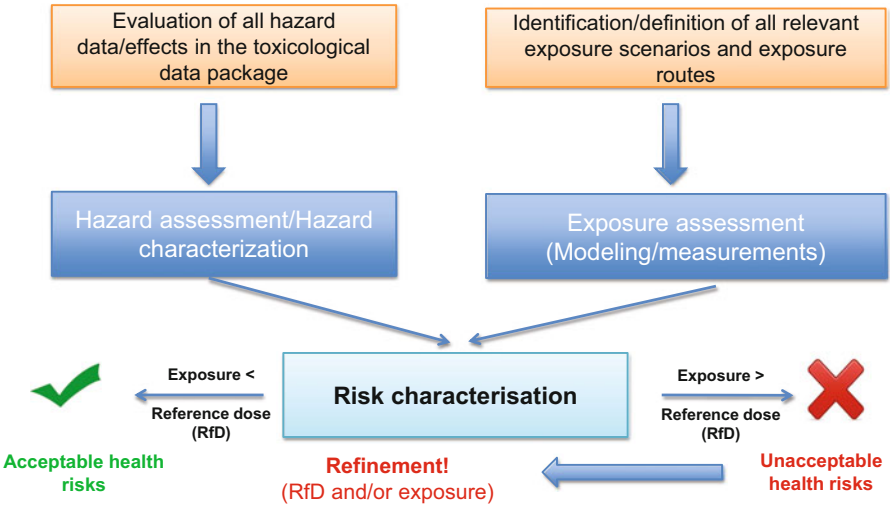

Fig. 1 Overview of the general principles of hazard/exposure assessment and risk characterization

assessment on the one side and the following exposure and risk assessment on the other are very similar for all registration/authorization procedures irrespective of the regulatory context.

Considering the two main pillars of regulatory toxicology, namely (a) hazard characterization/hazard assessment and (b) exposure evaluation and risk characterization, two basic questions need to be answered:

- Are the effects observed in *in vivo* rodent and/or nonrodent studies as well as in *in vitro* systems generally transferable to humans, i.e., are they relevant for humans?
- Are the intended uses of a substance in industrial, professional, and nonprofessional applications safe for humans and, depending on the applications and relevance, also for animals (livestock, pets), i.e., is the anticipated exposure as estimated with accepted modes or by measurements not associated with an unacceptable health risk?

The principles of hazard and exposure assessment and the associated risk characterization which are generally valid in all regulatory areas are depicted in Fig. 1 below:

Applied Regulatory Toxicology Using Biocides as an Example

The understanding and interpretation of effects observed in toxicological studies provide the basis for assessment and decision-making by regulators and risk assessors in the challenging field of regulatory toxicology.

To this end, scientific state-of-the-art knowledge and the expertise gathered in the interpretation of effects/findings in the various areas of toxicology are combined and integrated into the regulatory data requirements imposed by various horizontal legislations such as biocides, industrial chemicals, pharmaceutical drugs, and plant protection products, respectively.

The assessment of (active) substances with emphasis on the requirements on human health effects will be discussed in more detail using biocidal active substances and biocidal products containing these active substances as an example.

Overview of the Regulatory Process Toward the Approval of Biocidal Active Substances and Authorization of Biocidal Products

The approval of biocidal active substances and authorization of biocidal products in the area of the European Union follow a "two-step concept": Before a biocidal product can be placed on the market, the biocidal active substance needs to be approved first followed by the authorization of the biocidal product(s) containing the active substance (see Fig. 2).

The steps to be taken and requirements to be observed for approval of an active substance and authorizing a biocidal product are laid down in the "Biocidal Products Regulation" (BPR, Regulation (EU) No. 528/2012)

According to the definition of the BPR, an "**active substance**" means a substance or a microorganism that has an action on or against harmful organisms. These harmful organisms can be different types of bacteria, viruses, fungi, insects, or rodents which the active substance is efficacious against.

In general, it is not the biocidal active substances on their own but rather a **biocidal product** containing the active substances which is applied for the intended uses of the products. Within the meaning of Article 3(1)(a) of the BPR, a biocidal product means the following:

- Any substance or mixture, in the form in which it is supplied to the user, consisting of, containing or generating one or more active substances, with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action
- Any substance or mixture, generated from substances or mixtures which do not themselves fall under the first indent, to be used with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a

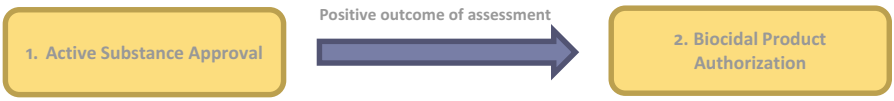

Fig. 2 Two-step process for biocidal active substance approval and biocidal product authorization

> controlling effect on any harmful organism by any means other than mere physical or mechanical action

However, there are some exceptions and applications where the active substance is applied in the pure form which means that in these particular cases the biocidal active substance and the biocidal product are identical.

Furthermore, there is a range of active substances which possess so called "dual uses," i.e., these active substances are applied and regulated under two or more horizontal legislations. For instance, an active substance used as an insecticide can be applied both as a biocidal active substance in stables, as an active substance to protect plants, or even as a veterinary drug for the treatment/protection of pets or livestock animals. The decision which legislations will become applicable for the respective products containing the active substance of interest depends on the claim made by applicants. For instance, if an active substance is intended to protect plants or plant products against insects, this substance is regulated under the plant protection regulation (Regulation (EC) No. 1107/2009), while the application of the same active substance in domestic settings for controlling an insect infestation in homes or to protect livestock animals used for food production is being dealt with under the BPR. Finally, there are some borderline cases where a clear decision on the applicable legislation cannot be made. These cases are to be presented to and discussed with the competent authorities responsible for different legislations. It could also happen that for a particular application, more than only one regulation is to be complied with by the prospective applicant.

Within the framework of active substance approvals under the BPR, the assessment of hazardous properties of the active substance and derivation of acceptable exposure levels (AELs) in relation to different exposure durations (e.g., acute, medium-term, and long-term AELs) are in the focus of the toxicology section of biocidal active substance dossiers. In contrast, the determination of the acute toxicity, primary irritation, and skin sensitization as well as the assessment of the potential endocrine-disrupting properties of nonactive substances contained in the biocidal products stands in the foreground in the toxicology section of biocidal product dossiers in addition to the demonstration of safe exposures and risks in the intended uses of biocidal products. With regard to exposure and risk assessments, it is also necessary to demonstrate a safe use of the active substance during the active substance approval process. In this case, however, the level of detail is not as extensive as during biocidal product authorization, and only one safe use is sufficient for active substance approval. Nevertheless, during biocidal product authorization, all intended uses of the concerned biocidal product(s) are to be considered and an acceptable risk to be shown either by modeling or exposure measurements in order to attain a positive decision during the authorization process.

In the context of the evaluation of biocidal active substances and the products containing these active substances, it is of particular note that the assessment of both active substances and the intended uses of biocidal products is always performed in connection with so-called product types (PTs) the active substances were originally notified for (see Commission Regulation (EC) No. 1451/2007 and Commission

Delegated Regulation (EU) No. 1062/2014 for the list of notified biocidal active substances and the product types they were notified for). An overview of the different product types for biocides including their scope and function is presented in Table 1 below:

When compared to the extent of human health hazard data required for biocidal products according to Annex III of the BPR, the data requirements for the human health hazard assessment of biocidal active substances as given in Annex II of the BPR are by far more comprehensive as all aspects of toxicity need to be addressed for a thorough and satisfactory appraisal of the toxicological profile. For this reason, the following subchapters are primarily dedicated to the illustration of the evaluation procedure and the toxicological data requirements in the process of the approval of biocidal active substances.

Approval Process for Biocidal Active Substances

Within the framework of the active substance approval process, the official assessment of biocidal active substances falls within the remits of an evaluating competent authority (eCA) of a EU member state. For this purpose, applicants for a biocidal active substance are required to prepare an active substance dossier observing the data requirements laid down in Annex II of the BPR and biocides dossier formats ("draft risk assessment report" for active substances) which are made available on the website of the ECHA (European Chemicals Agency).

Following submission of an application for active substance approval to the eCA, the dossier assessment process consists of two steps, namely **"validation"** and **"evaluation."**

In the validation step, the eCA investigates whether all data and information requirements in accordance with Annex II of the BPR have been provided for all section points of the active substance dossier. This step is, therefore, regarded as a completeness check, however, without performing a detailed assessment of the quality of the data submitted. Data gaps identified during the validation procedure need to be closed by the applicant within the time frames given in Art. 7 of the BPR and which normally should not exceed 90 days. Once the validation step has been passed and the dossier has been accepted by the eCA, the process moves on to the next stage which is the evaluation phase. This is the most important phase as the eCA will perform and in-depth evaluation of the quality/acceptability of the data submitted for all section points of the active substance including the acceptability of the methods and models used for exposure and risk assessments on the intended uses of the biocidal products containing the active substance.

It is of particular note that not all toxicological endpoints of Section 8 of Annex II of the BPR need to be addressed by study data, i.e., by the performance of *in vitro* and *in vivo* studies for the active substance of interest. The biocides regime offers the possibility of the preparation of "waivers" or "justifications for non-submission of data" in accordance with Article 6 (2)(a)–(c) and as detailed in Annex IV "General Rules for the Adaptation of the Data Requirements" of the BPR. This means that in justified cases, the generation of data on the active substance is not required if

Table 1 Overview of biocidal product types and their descriptions as referred to in Article 2(1) of the Biocidal Products Regulation (Regulation (EU) No 528/2012; 2012)

PT Number	Product type	Description
Main group 1: Disinfectants		
These product types exclude cleaning products that are not intended to have a biocidal effect, including washing liquids, powders, and similar products.		
1	Human hygiene	Human hygiene purposes:
		Application on or in contact with human skin or scalps for the primary purpose of disinfecting the skin or scalp
2	Disinfectants and algicides not intended for direct application to humans or animals	Disinfection of:
		Surfaces, materials, equipment, and furniture which are not used for direct contact with food or feeding stuffs
		Air, water not used for human or animal consumption, chemical toilets, waste water, hospital waste, and soil
		Use area(s):
		Swimming pools, aquariums, bathing, and other waters; air conditioning systems; and walls and floors in private, public, industrial areas, and in other areas for professional activities
		Algicides for treatment of swimming pools, aquariums, other waters, and for remedial treatment of construction materials
		Incorporation in textiles, tissues, masks, paints, and other articles or materials with the purpose of producing treated articles with disinfecting properties
3	Veterinary hygiene	Use area(s):
		Disinfectants, disinfecting soaps, oral or corporal hygiene products, or with antimicrobial function for veterinary hygiene purposes
		Disinfection of materials and surfaces associated with the housing or transportation of animals
4	Food and feed area	Uses area(s):
		Disinfection of equipment, containers, consumption utensils, surfaces or pipework associated with the production, transport, and storage or consumption of food or feed (including drinking water) for humans and animals.
		Impregnation of materials which may enter into contact with food

(continued)

Table 1 (continued)

PT Number	Product type	Description
5	Drinking water	Disinfection of human and animal drinking water
Main group 2: Preservatives		
Unless otherwise stated, these product types include only products to prevent microbial and algal development.		
6	Preservatives for products during storage	Use area(s):
		Preservation of manufactured products, other than foodstuffs, feeding stuffs, cosmetics or medicinal products, or medical devices by the control of microbial deterioration to ensure their shelf life
		Preservatives for the storage or use of rodenticide, insecticide, or other baits
7	Film preservatives	Use area(s):
		Preservation of films or coatings by the control of microbial deterioration or algal growth for protecting the initial properties of the surface of materials or objects such as paints, plastics, sealants, wall adhesives, binders, papers, and art works
8	Wood preservatives	Use area(s):
		Preservation of wood, from and including the saw-mill stage, or wood products by the control of wood-destroying or wood-disfiguring organisms, including insects in both preventive and curative applications
9	Fiber, leather, rubber, and polymerized materials preservatives	Use area(s):
		Preservation of fibrous or polymerized materials, such as leather, rubber or paper, or textile products by the control of microbiological deterioration
10	Construction material preservatives	Use area(s):
		Preservation of masonry, composite materials, or other construction materials other than wood by the control of microbiological and algal attack
11	Preservatives for liquid-cooling and processing systems	Use area(s):
		Preservation of water or other liquids used in cooling and processing systems by the control of harmful organisms such as microbes, algae, and mussels
12	Slimicides	Use area(s):
		Prevention or control of slime growth on materials, equipment, and structures, used in industrial processes, e.g., on wood and

(continued)

Table 1 (continued)

PT Number	Product type	Description
		paper pulp, porous sand strata in oil extraction
13	Working or cutting fluid preservatives	Use area(s):
		Control of microbial deterioration in fluids used for working or cutting metal, glass, or other materials
Main group 3: Pest control		
14	Rodenticides	Use area(s):
		Control of mice, rats, or other rodents by means other than repulsion or attraction
15	Avicides	Use area(s):
		Control of birds by means other than repulsion or attraction
16	Molluscicides, vermicides, and products to control other invertebrates	Use area(s):
		Control of molluscs, worms, and invertebrates not covered by other product types by means other than repulsion or attraction
17	Piscicides	Use area(s):
		Control of fish by means other than repulsion or attraction
18	Insecticides, acaricides, and products to control other arthropods	Use area(s):
		Control of arthropods (e.g., insects, arachnids, and crustaceans) by means other than repulsion or attraction.
19	Repellents and attractants	Use area(s):
		Control harmful organisms
		invertebrates such as fleas
		vertebrates such as birds, fish, and rodents
		by repelling or attracting, including those that are used for human or veterinary hygiene either directly on the skin or indirectly in the environment of humans or animals
20	Control of other vertebrates	Use area(s):
		Control of vertebrates other than those already covered by the other product types of this main group
		by means other than repulsion or attraction.
Main group 4: Other biocidal products		
21	Antifouling products	Use area(s):
		Control of growth and settlement of fouling organisms (microbes and higher forms of plant or animal species) on vessels,

(continued)

Table 1 (continued)

PT Number	Product type	Description
		aquaculture equipment, or other structures used in water
22	Embalming and taxidermist fluids	Use area(s):
		Disinfection and preservation of human or animal corpses, or parts thereof.

1. **Testing Does Not Appear to Be Scientifically Necessary**
   - By using already existing data which on their own, or in the context with other available independent sources of information and applying a WoE approach, are sufficiently reliable for classification and labeling and the key parameters are adequately covered.
   - By using a grouping/read-across approach to reliable data on substances of similar structure and properties ("reference substances"); the validity of such an approach needs to be proven by the comparison of data on available reference points.
   - If available data of sufficient reliability/validity cover the exposure durations foreseen in the intended applications of biocidal products containing the concerned active substance.
   - If QSAR (qualitative or quantitative structure-activity relationship) methods provide reliable and adequate information on the endpoint to be waived.
   - If it can be shown by reliable data that due to its high reactivity and associated rapid degradation, an active substance does not become systemically available; in this case, investigations directed to local effects may be sufficient.
2. **Testing Is Technically Not Possible**
   - If based on the physico-chemical properties of the substance, study data cannot be established, e.g., oral/dermal testing of highly volatile substances or testing of highly reactive or unstable substances in general.

Although a third-waiving option based on exposure considerations is provided for in the BPR ("Product-Tailored Exposure-Driven Testing"), this option is rarely applicable in the biocides area as the application of biocides in general will in the majority of cases be associated with an exposure of various population subgroups. Use of this option can, therefore, only be made on a case-by-case basis considering details of the intended biocidal application(s).

Evaluation and Defense of Biocidal Active Substances During the Approval Process

The most challenging part of the active substance approval process is represented by the evaluation phase with a focus on the defense of the active substance dossier as submitted by an applicant. This stage involves both the applicant and

the selected eCA as well as the corresponding experts of all EU biocides authorities which are explained in more detail in the following:

1. According to BPR Article 8 "Evaluation of applications," the eCA is required to complete its evaluation of the active substance within 365 days of the validation of an application. In case the eCA had further requirements during the evaluation phase, e.g., the need to revise the assessment of study reports or exposure and risk assessments, an extension could be granted which usually shall not exceed a "stop of the clock" period of 180 days.
2. After completion of the evaluation of the active substance dossier by the eCA, it prepares a "draft competent authority report" (dCAR). In this dCAR, the results of the evaluation are summarized. In a first step, the dCAR and the comments of the eCA are sent to the applicant. The applicant is granted a period of 30 days to comment on and clarify outstanding issues identified by the eCA by submitting scientifically robust reasonings in the form of, e.g., expert statements/position papers.
3. Thereafter, the conclusions of the evaluation of the eCA together with a discussion table listing unresolved comments/open points identified during the evaluation are sent to the European Chemical Agency (ECHA) where they are subject to a peer review by the "Biocidal Products Committee" (BPC). The BPC Tox Working Group (WG) comprising human health experts of all EU biocides authorities is responsible for the assessment of human health hazards, exposure and risk characterization of the concerned active substance.
4. The conclusions of the BPC Tox WG and the open issues which could not be solved in the WG discussions are passed on to the Biocidal Products Committee (BPC) which aims at a conclusion on all section points of the active substance. The results of the BPC discussions will be adopted in a BPC Opinion which contains also further elements to be taken into account when authorizing products in the products types applied for and which do not prevent the active substance from being approved. The decisions made on the assessment of the active substance and the reasons for the conclusions drawn are summarized in the BPC Opinion on the active substance in its respective products types. The BPC phase for discussing the evaluation and concluding on the approval or nonapproval of the active substance shall not exceed a period of 270 days.
5. The outcomes of the BPC conclusions will be implemented by the eCA in a revised version of the Assessment Report. If in the subsequent comitology procedure of the Standing Committee on Biocidal Products of the European Commission there are no objections on the decisions made in the BPC, the active substance approval process is finalized. The BPC opinion together with the Assessment Report of the active substance in its respective product types will be published on the ECHA website and the active substance included in the Union list of approved active substances.

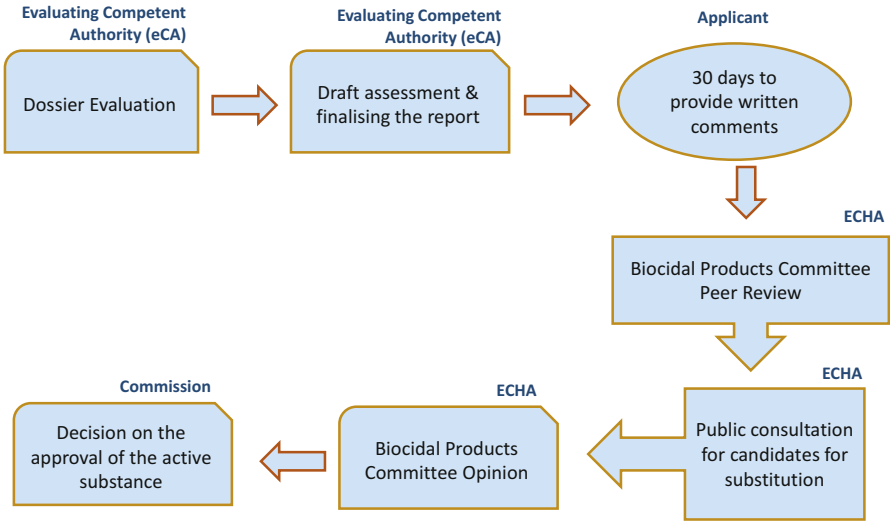

Fig. 3 Overview of the evaluation process for biocidal active substances by the eCA, Applicant, BPC, and the Commission (https://echa.europa.eu/regulations/biocidal-products-regulation/approval-of-active-substances/existing-active-substance/evaluation-process-for-active-substances-under-the-review-programme)

An overview of the active substance approval process is depicted in Fig. 3 below.

A biocidal active substance is approved for a maximum period of 10 years. Thereafter, the approval is to be renewed on the basis of the data/studies originally submitted and taking into a consideration new data requirements which may have been drawn up in the meantime.

If active substances reveal certain hazardous properties, the exclusion criteria according to Art. 5(1) or the criteria for candidates for substitution (CfS) according to Art. 10(1) of the BPR may apply. Most importantly, active substances fulfilling the exclusion criteria will in principle not be approved.

Both the criteria for exclusion and substitution are strictly based on the intrinsic hazardous properties in combination with the use without taking into consideration any aspects related to exposure and risks.

An active substance will be considered as meeting the criteria for exclusion or substitution if any of the following hazardous properties are identified during evaluation:

1. **Active Substances Fulfilling the Exclusion Criteria**
   - Carcinogens, mutagens, and reprotoxic substances categories 1A or 1B according to the CLP Regulation
   - Endocrine disruptors
   - Persistent, bioaccumulative, and toxic (PBT) substances
   - Very persistent and very bioaccumulative (vPvB) substances
2. **Active Substances Which Are Candidates for Substitution**
   - Active substances fulfilling the exclusion criteria.
   - Active substances classified as a respiratory sensitizer.

- Toxicological reference values of the active substance are significantly lower than those of the majority of approved active substances for the same product type and use.
- Active substances meeting two of the PBT criteria.
- Active substances causing concerns for human or animal health and for the environment even with very restrictive risk management measures.
- Active substances containing a significant proportion of nonactive isomers or impurities.

Toxicological Data Requirements of Biocidal Active Substances According to the Provisions of the Biocidal Products Regulation

One major pillar in the approval process of biocidal active substances is represented by the toxicological data package to be established in relation to the human health hazard assessment with the aim to enable a) classification and labeling of the substance, and b) derivation of tolerable reference values for systemic and local effects.

The data package to be prepared is comprised of studies on the following human health-related endpoint studies (not exhaustive):

- Acute toxicity studies (oral, dermal, and inhalation)
- Primary irritation studies (skin and eye)
- Skin and respiratory sensitization
- Repeated dose toxicity (RDT) studies:28-/90-day studies in rodents (e.g., rats), 90-day/1 year studies in nonrodents (mostly dogs)
- Carcinogenicity studies in rodents (rats and mice)
- Developmental toxicity and reproductive toxicity studies ("DART" studies)
  - Reproductive toxicity studies: Two-generation reproductive toxicity studies (TGRTS) or extended one generation study (EOGRTS) in rats
  - Developmental toxicity and teratogenicity studies in rats and rabbits
- Acute/subchronic neurotoxicity and delayed neurotoxicity studies
- Developmental neurotoxicity (DNT) and developmental immunotoxicity (DIT) studies as (optional) parts of the EOGRTS
- Dedicated *in vitro* and *in vivo* studies on the elucidation of the potential endocrine-disrupting (ED) properties with a focus on endocrine activity and related adverse effects

The toxicological data requirements distinguish endpoints pertaining to the Core Data Set (CDS) or Additional Data Set (ADS).

Data assigned to the CDS are to be regarded as basic data which should, in principle, be provided for all active substances. In case there are grounds for deviating from the CDS and an endpoint is waived, a scientifically sound explanation on the nonsubmission of the respective data needs to be provided.

Data, assigned to the ADS, are to be provided by taking into account, for instance, the physical and chemical properties of the substance, existing data, and information which is part of the CDS, the types of products in which the active substance will be used and the exposure patterns related to these uses.

With the application of the most recent "ECHA/EFSA Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009," there is now a need to assess the potential endocrine-disrupting properties of biocidal active substances before a final conclusion on the approval or nonapproval of an active substance can be drawn. As a consequence, the data requirements in Annex II and III of the current version of the BPR need to be adapted to the scientific and technical progress in relation to the determination of endocrine-disrupting properties both for active substances and biocidal products (for details, please refer to most recent Competent Authority document "CA-Feb20-Doc.3.1.b – Rev BPR annexes.docx").

The most profound revisions on the toxicological data requirements in Section 8 of Annex II of the BPR concern reproductive toxicity (8.10) and the newly introduced endpoint endocrine disruption (8.13.3).

For reproductive toxicity examination, preference is given to the EOGRTS using cohorts 1A and 1B for effects on reproduction/fertility and effects on a pup/litter level. In addition, the EOGRTS offers a number of advantages in comparison to the TGRTS as it assesses in addition to effects on the male and female reproductive system more toxicological effects linked to endocrine-disrupting modes of action.

In relation to the (separate) assessment of endocrine-disrupting properties, a whole battery of *in vitro* and *in vivo* tests needs to be performed comprising studies on the mechanism of action and adversity.

Another endpoint which is affected by the revision of the Annex II concerns studies on the investigation of potential developmental neurotoxicity (DNT) which will be changed from an ADS to a CDS and becomes, thus, a mandatory data requirement in the proposal for an update of Annex II of the BPR. This investigation shall be integrated as cohorts 2A and 2B of the EOGRTS as the preferred study type for the examination of potential reproductive toxicity.

Table 2 summarizes the data requirements and specific rules for adaptation of the selected endpoints mentioned above.

Assessment of Potential Endocrine Disruption

WHO Definition of an Endocrine Disruptor

With the entry into force and application of the ECHA/EFSA guidance document on the assessment of potential endocrine-disrupting (ED) properties of active substances used in biocidal and plant protection products, the assessment and conclusion on the potential ED properties became a mandatory data requirement within the scope of the approval of biocidal active substances.

The basis of the determination of potential ED properties of biocidal active substances is based on the WHO definition of an endocrine disruptor (WHO 2002). According to this definition, a substance shall be considered as having endocrine-disrupting properties that may cause adverse effect in humans if it is a substance that meets all of the following criteria, unless there is evidence demonstrating that the adverse effects identified are not relevant to humans:

Table 2 Selection of proposed amendments on the toxicological data requirements for active substances according to Annex II of the BPR with a focus on CDS for reproductive toxicity, developmental neurotoxicity, and endocrine disruption (CA-Feb20-Doc.3.1.b – Rev BPR annexes.docx; CIRCA BC, 2020)

Endpoint	Specific rules for adaptation from column 1
8.10 Reproductive toxicity	The studies do not need to be conducted if:
For evaluation of consumer and animal safety of active substances that may end up in food or feed, it is necessary to conduct toxicity studies by the oral route.	The substance meets the criteria to be classified as a genotoxic carcinogen (classified both as germ cell mutagen category 2, 1A or 1B, and carcinogenic category 1A or 1B), and appropriate risk management measures are implemented including measures related to reproductive toxicity
	The substance meets the criteria to be classified as a germ cell mutagen category 1A or 1B, and appropriate risk management measures are implemented including measures related to reproductive toxicity
	The substance is of low toxicological activity (no evidence of toxicity seen in any of the tests available provided that the dataset is sufficiently comprehensive and informative); it can be proven from toxicokinetic data that no systemic absorption occurs via relevant routes of exposure (e.g., plasma or blood concentrations below detection limit using a sensitive method and absence of the substance and of metabolites of the substance in urine, bile, or exhaled air), and the pattern of use indicates there is no or negligible significant human or animal exposure
	The substance meets the criteria to be classified as reproductive toxicity category 1A or 1B: may damage fertility (H360F), and the available data are adequate to support a robust risk assessment, then no further testing for sexual function and fertility will be necessary. A full justification must be provided and documented if investigations for developmental toxicity are not conducted or
	The substance is known to cause developmental toxicity, meeting the criteria for classification as reproductive toxicity category 1A or 1B: may damage the unborn child (H360D), and the available data are adequate to support a robust risk assessment, then no further testing for developmental toxicity will be necessary. A full justification must be provided and documented if investigations for sexual function and fertility are not conducted
	Notwithstanding the provisions of this column of this subsection, studies on reproductive toxicity may need to be conducted to obtain

(continued)

Table 2 (continued)

Endpoint	Specific rules for adaptation from column 1
	information on endocrine-disrupting properties as laid down in 8.13.3.1.
8.10.2 Extended One-Generation Reproductive Toxicity Study (OECD TG 443),	A two-generation reproductive toxicity study conducted in accordance with OECD TG 416 (adopted 2001 or later) or equivalent information shall be considered appropriate to address this information requirement, if the study is available and was initiated before . . . (*OJ please insert the date of application of this amending Regulation*).
with cohorts 1A and 1B and extension of cohort 1B to include the F2 generation with the aim to produce 20 liters per dose group, F2 pups must be followed to weaning and investigated similarly as F1 pups. Rat is the preferred species, and oral route of administration is the preferred route.	
The highest dose level should be based on toxicity and selected with the aim to induce reproductive and/or other systemic toxicity.	
8.10.3 Developmental neurotoxicity	The study shall not be conducted if the available data:
Developmental Neurotoxicity Study in accordance with OECD TG 426, or any relevant study (set) providing equivalent information, or by cohorts 2A and 2B of an Extended One-Generation Reproductive Toxicity study (OECD TG 443) with additional investigation for cognitive functions	Indicate that the substance causes developmental toxicity and meets the criteria to be classified as toxic for reproduction category 1A or 1B: may damage the unborn child (H360D)
	Are adequate to support a robust risk assessment
8.13.3 Endocrine disruption	Where sufficient weight of evidence to conclude on the presence or absence of a particular endocrine-disrupting mode of action is available:
The assessment of endocrine disruption shall comprise the following tiers:	Further testing on vertebrate animals for that effect shall be omitted for that mode of action
(a) An assessment of the available information from the following studies and any other relevant information, including *in vitro* and *in silico* methods:	Further testing not involving vertebrate animals may be omitted for that mode of action.
(i) 8.9.1 A 28-day oral study in rodents (OECD TG 407)	In all cases, adequate and reliable documentation shall be provided.
(ii) 8.9.2 A 90-day oral study in rodents (OECD TG 408)	
(iii) 8.9.4 A repeated dose oral study in nonrodents (OECD TG 409)	
(iv) 8.10.1 A prenatal developmental toxicity study (OECD TG 414)	
(v) 8.10.2 An extended one-generation reproductive toxicity study (OECD TG 443) or two-generation reproductive toxicity study (OECD TG 416)	
(vi) 8.10.3 A developmental neurotoxicity study (OECD TG 426)	

(continued)

Table 2 (continued)

Endpoint	Specific rules for adaptation from column 1
(vii) 8.11.1 A combined carcinogenicity study and long-term repeated dose toxicity study (OECD TG 451-3)	
(viii) A systematic review of the literature including studies on mammals and nonmammalian organisms	
(b) If there is any information suggesting that the active substance may have endocrine-disrupting properties, or if there is incomplete information on key parameters relevant for concluding on endocrine disruption, then additional information or specific studies shall be required to elucidate any of the following:	
(1) the mode or the mechanism of action	
(2) potentially relevant adverse effects in humans or animals	
For evaluation of consumer safety of active substances that may end up in food or feed, it is necessary to consider the oral route and conduct animal studies by the oral route.	
8.13.3.1 Specific additional studies to investigate potential endocrine-disrupting properties may include, but are not limited to the following:	
(a) the mammalian toxicity studies listed in 8.13.3 (a)	
(b) the *in vitro* assays:	
(i) Estrogen receptor transactivation assay (OECD TG 455)	
(ii) Androgen receptor transactivation assay, (OECD TG 458)	
(iii) H295R steroidogenesis assay (OECD TG 456)	
(iv) the Aromatase assay (human recombinant) OPPTS 890.1200	
(c) Uterotrophic bioassay in rodents (OECD TG 440) and Hershberger bioassay in rats (OECD TG 441)	
(d) Pubertal development and thyroid function in intact juvenile or peripubertal male rats (OPPTS 890.1500)	
The decision to carry out studies in mammals shall be taken based on all available information, including a systematic review of the literature (including information on endocrine-disrupting effects in nontarget organisms) and the availability of suitable in silico or *in vitro* methods.	

- Observation of an adverse effect in an intact organism or its progeny (change in the morphology, physiology, growth, development, reproduction, or life span of an organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences).
- Identification of an endocrine mode of action (altering the function(s) of the endocrine system).
- Adverse effect is a consequence of the endocrine mode of action.

Scientific Criteria for the Determination of Endocrine-Disrupting Properties

Scientific criteria for the determination of endocrine-disrupting properties were not available within the process of the approval of biocidal active substances until recently. Therefore, a major task consisted in the definition of such criteria which became available with the application of the Commission Delegated Regulation (EU) No. 2017/2100.

The scientific criteria differentiate between criteria for the determination of potential ED properties in humans in Section A and on nontarget organisms in part B of the regulation.

The following main elements which need to be taken into consideration in the process of the determination of potential ED properties in humans comprise the following aspects:

- Gathering of all relevant scientific ED-related data (in vivo, in vitro, in silico)
- Assessment of all relevant scientific ED-related data applying a WoE approach considering:
  - Positive/negative results
  - Relevance of study design, quality, and consistency of data
  - Exposure route and toxicokinetics
  - Limit dose/MTD (maximum tolerated dose) effects
  - Adverse effects of nonspecific consequences of toxicity which are not to be considered/not relevant for the determination of potential ED properties

All of the above listed criteria are to be met in order to conclude on the presence or absence of potential ED properties of a biocidal active substance.

Main Elements of the ECHA/EFSA Guidance for the Identification of Endocrine Disruptors

Since the assessment of potential ED properties was not part of biocidal active substance dossiers before the application of the ED guidance document and as in most cases no attention was paid to possible endocrine-mediated effects, all study data and information submitted in relation to active substance approval have to be reassessed with a particular focus on potential ED effects.

In the ECHA/EFSA guidance document, focus is placed on ED properties based on "EATS (Estrogen, Androgen, Thyroid and Steroid)-mediated effects." To date, these endpoints are best understood from the mechanistic point of view and

standardized *in vitro/in vivo* test methods and guidelines are available on these endpoints with broad scientific agreement on the interpretation of the effects observed on the investigated parameters. The available test methods are provided for in the OECD Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (OECD GD 150) which includes the "OECD Conceptual Framework (OECD CF) for Testing and Assessment of Endocrine Disruptors" providing a grouping of the studies into five levels according to the kind of information provided.

For the assessment of the potential ED properties of an active substance, a grouping is performed in the evaluation of the scientific evidence when identifying substances with ED properties which comprises:

- ***In vitro* mechanistic:** *In vitro* parameters of OECD CF level 2 providing information on a potential endocrine mechanism (i.e., receptor binding/activation, interaction with hormone production)
- ***In vivo* mechanistic:** *In vivo* parameters of OECD CF level 3 providing information on usually nonadverse endocrine activity
- **EATS-mediated:** *In vivo* parameters contributing to the evaluation of adversity, while at the same time they are also considered indicative of an EATS mode of action and imply underlying *in vivo* mechanistic information
- **Sensitive to, but not diagnostic of, EATS:** *In vivo* parameters from OECD CF levels 3–5 which contribute to the evaluation of adversity, but are not sufficiently indicative on their own of any one of the EATS modalities

In accordance with the ECHA/EFSA ED guidance document, the ED assessment comprises the five following stages toward assessment of ED properties which are briefly summarized below and are based on the strategy for the assessment of potential ED properties of active substances used in biocidal and plant protection products as provided for in the ED guidance document (Fig. 4):

1. **Gathering of All Relevant Information/Parameters Relevant for ED Assessment**
   - *In vivo/in vitro* mechanistic data (Levels 2 + 3 of OECD CF)
   - EATS-mediated parameters (Levels 4 + 5 of OECD CF)
   - Sensitive to, but not diagnostic of, EATS parameters (Levels 3–5 of OECD CF)
2. **Assembling, Assessing, and Integrating the Lines of Evidence**
   - Assessment of adverse effects with a view to ED relevance ("EATS-mediated parameters" and "sensitive to, but not diagnostic of, EATS parameters")
   - Assessment of available information on ED activity (*in vivo/in vitro* mechanistic data, EATS parameters)
3. **Initial Analysis of the Evidence**
   - Judgement if EATS-mediated parameters/ED activities are sufficiently investigated and a potential biological plausible link between (EATS-mediated) adversity and potential ED activity is established.

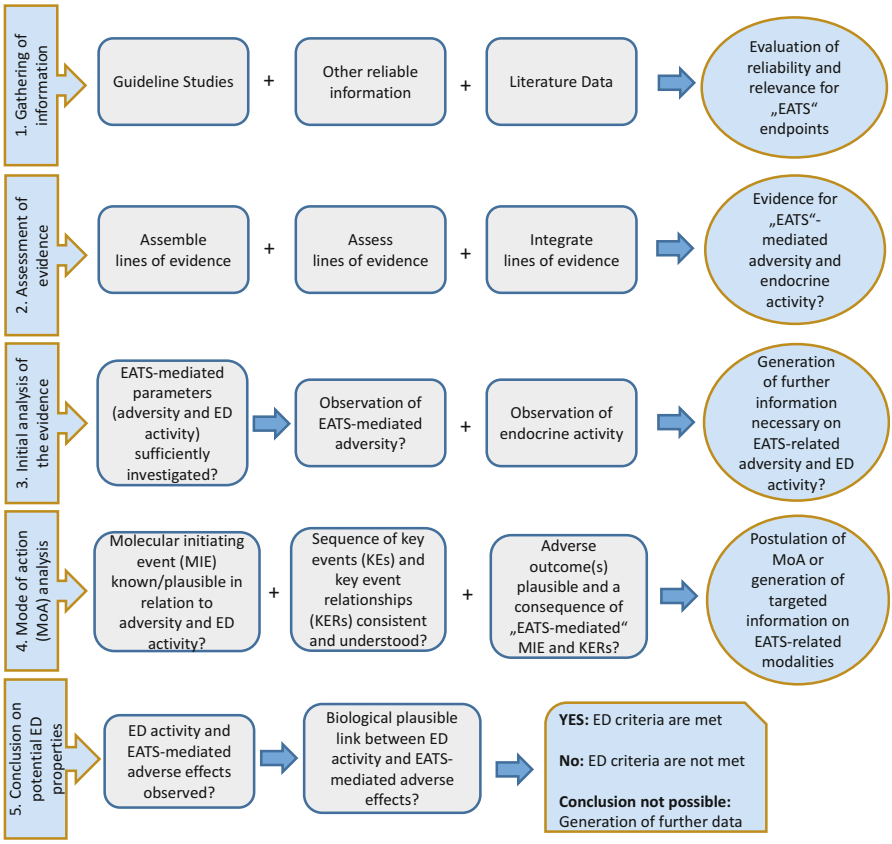

Fig. 4 Flowchart on the assessment strategy for potential ED properties of active substances used in biocidal and plant protection products

4. **Mode of Action Analysis**
   - If EATS-mediated adversity and/or ED activity is observed, an in-depth analysis of the mode of action and the cascade MIE ➔ KE1-n ➔ AO is needed
     (MIE: molecular initiating event; KE: key event; AO: Adverse outcome).
5. **Conclusion Whether the Substance Meets the ED Criteria**
   - A conclusion on a positive/negative ED potential is always required. If a conclusion is not possible, the need and a strategy for the generation of further *in vitro/in vivo* data are to be considered.

The reassessment of all available information with a particular focus on ED properties and establishment of a biologically plausible link between potential ED activity and observed adverse effects is associated with high efforts and requires a high level of expertise in order to:

(a) Reevaluate and assess the available study data

(b) Postulate/elucidate a potential endocrine mode of action
(c) Decide on further ED-specific testing taking into account integrated approaches for testing and assessment
(d) Draw a final conclusion on the absence or presence of potential ED properties of the active substance

Derivation of Acceptable Exposure Levels (AELs)/Concentrations (AECs) for Biocidal Active Substances

Besides the demonstration of safe uses in the intended application of biocidal products and the active substances contained therein, the derivation of acceptable reference values both for systemic and local effects represents the final objective of the toxicological hazard characterization process during active substance approval. The deduction of a reference value for an active substance is a crucial step in the evaluation procedure as the reference values constitutes a crucial determinant in the risk characterization process.

Depending on the effects observed in the available or generated toxicological data package, it needs to be differentiated between substances exerting a systemic and/or local mode of action. According to the terminology for biocides, acceptable exposure levels (AELs) are derived for substances with systemic effects, while for local effects, acceptable exposure concentrations (AECs) are derived.

Usually, the most relevant and sensitive endpoints identified in the toxicological data package serve as the point of departure (i.e., no-observed-adverse-effect-level, NOAEL) for reference value deduction including the application of uncertainty factors to correct the NOAEL for inter-/intraspecies variabilities in toxicodynamic and toxicokinetics and to adjust for bioavailability and/or exposure duration. Further uncertainty factors may have to be applied for the extrapolation from a lowest-observed-adverse-effect-level (LOAEL) to a no-observed-adverse-effect-level if only a LOAEL could be derived from the data/studies available. Most importantly, if more than one "key study" is available for reference value setting, a weight-of-the evidence approach is used with a view to the identification of the most relevant and most sensitive point of departure.

This process affords a high level of expertise and experience by undertaking considerations on, e.g., dose setting, dosing duration, effects observed at or above the maximum tolerated dose (MTD effects), and relevance of the findings made for humans. Furthermore, with a view to the exposure and risk assessment in the intended uses of an active substance which exerts systemic effects, the foreseen exposure duration for an application may trigger the need for the derivation of acceptable exposure levels for different time frames. Exposure-related information is, therefore, crucial for the adjustment of human health risk assessment to varying time frames for professional as well as consumer exposure.

With regard to the derivation of reference values for local effects ("AECs"), the regulatory process differentiates between

– Local effects via the dermal route and
– Local effects directed toward the respiratory tract

The common understanding in the derivation of AECs for biocidal active substance is that these effects are concentration-related rather than dosage-related. In general, if local effects are independent of metabolic processes, considerations on toxicokinetics for the correction of the NOAEC (i.e., a no-observable-adverse-effect concentration) as the starting point are not required, and only uncertainty factors to account for inter- and intraspecies differences in toxicodynamics are applied. However, in the course of the defense of active substances, a point of discussion is always related to the uncertainty factor to be applied for the derivation of reference values for local effect via the inhalation route. Based on the inhalation NOAEC, the following types of correction are considered in this particular case:

– Inter- and intraspecies differences for toxicodynamics
– Intraspecies differences in toxicokinetics
– Exposure duration in the study vs. anticipated exposure duration during application

Especially the need to correct the NOAEC for exposure duration has to be carefully considered and to be decided upon on a case-by-case basis taking into account the available data.

The type of assessment/uncertainty factor as well as the extrapolation factors usually applied in the derivation of reference values for systemic and local effects of biocidal active substances is given in Table 3:

Finally, after having identified the most appropriate point of departure(s) and following a correction of the starting point(s) by the application of the required uncertainty factors, the corresponding reference values, i.e., AELs/AECs, are derived. Table 4 below provides an overview of the different types of reference values for biocidal active substances and their relationship to diverse categories of users.

The type of AEL derived is largely depending on the intended application of the active substance and the category of users applying the biocidal products containing the active substance. In Table 5 below, the relationship between duration of human exposure and the studies required for hazard identification and derivation of the relevant points of departure as retrieved from the Guidance on BPR: Volume III Parts B+C (Version 4.0 December 2017), are summarized.

More details and specific needs in relation to the derivation of acceptable exposure levels and concentrations as well as uncertainty/correction factors to be applied can be found in Volume III, Part B+C of the ECHA Guidance on the assessment for human health (Guidance on the Biocidal Products Regulation 2017).

Table 3 Default uncertainty/correction factors in the derivation of AELs/AECs for biocidal active substances

Type of uncertainty	Default uncertainty factor	Remark
Interspecies differences – systemic effects:		Allometric scaling factors:
Toxicodynamics	2.5	Dog: 1.4
Toxicokinetics (allometric scaling)	1.4–7 (species specific)	Rat: 4
		Mouse: 7
Intraspecies differences – systemic effects:		Adjustment of allometric scaling factors in humans if substance-specific data are available
Toxicodynamics	3.16	
Toxicokinetics (allometric scaling)	3.16	
Interspecies differences – local dermal effects:		Factor of 2.5 is required if dermal effects are dependent on local metabolism
Toxicodynamics	1–2.5	
Toxicokinetics (allometric scaling)	1	
Intraspecies differences – local dermal effects:		Expert judgment
Toxicodynamics	1–3.16	
Toxicokinetics (allometric scaling)	1–3.16	
Interspecies differences – local effects respiratory tract:		Expert judgment in case workplace measurements in humans is available
Toxicodynamics	2.5	
Toxicokinetics (allometric scaling)	1	
Intraspecies differences – local effects respiratory tract:		Expert judgment in case workplace measurements in humans is available
Toxicodynamics	3.16	
Toxicokinetics (allometric scaling)	3.16	
Exposure duration (systemic and local inhalation effects)		
Subchronic to chronic	2	
Subacute to subchronic	3	
Subacute to chronic	6	
LOAEL-NOAEL extrapolation	2–10	Expert judgment based on dose-response considerations applying weight of the evidence approach

Table 4 Overview of toxicological reference values for active substances in relation to the concerned user categories

Reference value type	User categories
Systemic effects	
$AEL_{acute/short-term}$	Nonprofessional users/bystanders
$AEL_{medium-term}$	Industrial/professional/ nonprofessional users
$AEL_{long-term}$	Industrial/professional users
(AELs needed in case of systemic effects following dermal/ inhalation exposure; for oral exposure, the acceptable intake (ADI) is preferred although an AEL could be used as surrogate following correction of oral exposure by systemic availability)	
Local effects	
AEC_{dermal}	Industrial/professional/ nonprofessional users/bystanders
AEC_{inhal}	Industrial/professional/ nonprofessional users/bystanders
(AECs are needed in case the active substance exerts local effects via the dermal and/or inhalation routes of exposure)	

Table 5 Relationship between duration of human exposure and the studies required for hazard identification and derivation of the relevant points of departure

Anticipated duration of human exposure	Relevant toxicity studies to be selected	Relevant point(s) of departure for AEL derivation
≤24 h	Single-dose studies designed to determine points of departure[a] or repeated dose studies demonstrating relevant acute effects, e.g.,	Toxic effects relevant for acute exposure
	acute neurotoxicity studies	
	28-d/90-d repeated-dose studies, acute effects	
	developmental toxicity studies, acute effects	
>24 h – 3 months (max. 6 months)	Repeated-dose studies designed to determine dose descriptors, e.g.,	Toxic effects relevant for medium-term exposure
	28-d/90-d repeated-dose studies	
	90-d neurotoxicity studies	
	12-m dog studies, depending on the nature of effects	
	developmental toxicity studies	
	2-generation studies	
>6 months (min. 3 months)	Chronic studies or repeated dose studies designed to determine dose descriptors and demonstrating relevant chronic effects, e.g.,	Toxic effects relevant for long-term exposure
	18-m/24-m chronic/carcinogenicity studies	

[a]Data from LD_{50} studies can be considered supportive if appropriate acute effects were investigated

Exposure Assessment and Risk Characterization for Biocides

General Principles on Exposure and Risk Assessment

The focus of the approval process of biocidal active substances lies on the assessment of the intrinsic hazardous properties by gathering and evaluating all available data and study information of the concerned active substance to enable the following:

(a) Classification and labeling of the active substance
(b) Derivation of tolerable reference values for systemic and/or local effects

A further important cornerstone in the approval of biocidal active substances constitutes the demonstration of safe uses in the product type-dependent intended applications of the biocidal products containing the active substance.

This process encompasses the identification of the relevant exposure scenarios, exposure assessment, and risk characterization with the objective to demonstrate that the estimated exposure is acceptable and does not lead to an exceedance of the relevant reference values derived for the active substance from the most relevant study and most sensitive animal species.

The scope of exposure and risk assessments in general is described by the following elements:

- Estimation of the potential dermal, inhalation, and, where relevant, the oral exposure following application of a substance in its intended use(s)
- Characterization and identification of the potential risks associated with handling and use, i.e., comparison of the estimated exposure with a "tolerable exposure level" derived for the active substance based on the most sensitive endpoint from the most relevant toxicity study in experimental animals
- Demonstration of safe uses and absence of unacceptable health risks for workers, consumers, and/or the general population (including the young and elderly persons)

During active substance approval, however, not all uses which the biocidal product is intended to be applied for need to be addressed. In the context of the safe use concept, it is sufficient at this stage to show that one intended use is safe from the risk assessment point of view. However, all other uses of the biocidal product which were not covered in the active substance dossier need to be addressed at the biocidal product authorization stage at the latest.

Irrespective of the approach chosen during active substance approval in relation to the intended uses, the following stepwise approach applies in general to all exposure assessments which need to be performed on the biocidal product containing the active substance:

1. Determination of the relevant product types and formulations the biocidal active substance is intended to be used for

2. Identification of the exposed type of population which could comprise the following:
   - Industrial users
   - Professional users
   - Nonprofessional/amateur users
   - The general public including children and the elderly
3. Identification of the exposure scenarios which are composed of the following:
   - Use pattern for each identified population subgroup, i.e., definition of the different tasks/working procedures to be performed for a specific application
   - Routes of exposure (e.g., inhalation, dermal, and oral route)
   - Frequency and duration of the concerned use(s)
   - Determination of primary and secondary exposure scenarios
4. Estimation of exposure by the following:
   - Using exposure models agreed upon by the human exposure working groups of the BPC
   - Building of justified generic exposure scenarios including a reasoning on their applicability
   - Using available measured data for specific use scenarios if relevant, reliable, and valid

With a view to the performance of exposure assessments for biocides, a differentiation needs to be made between primary and secondary exposure. This difference will have a decisive impact on the type of population exposed and the feasibility of the implementation of risk mitigation measures, i.e., the possibility of the use of personal/respiratory protective equipment (PPE/RPE) such as gloves, coveralls, or respirators, respectively.

Primary Exposure
Primary exposure is related to those individuals applying the active substance and the biocidal product containing it in its intended application(s). In this respect, a refinement of the exposure estimations by taking into consideration PPE/RPE is only possible for industrial or professional workers. This group of operators is usually trained in the applications to be performed and have knowledge on worker protection. In contrast, nonprofessional workers apply biocidal products less frequently, are less skilled, and usually have no or only little knowledge on the use of PPE/RPE. For this reason, any kind of PPE/RPE can usually not be taken into account in the estimation of the primary exposure of nonprofessional users.

Secondary Exposure
Secondary exposure will be encountered during and after application by those individuals not directly involved in the application itself. However, also the individual having performed the application could additionally be secondarily exposed once the application has been completed and treated areas are reentered or surfaces are being touched for instance. In these particular exposure situations, exposure-reducing measures such as PPE/RPE will not be taken into account

unless specific instructions have been released in industrial and/or professional work areas.

Exposure Modeling for Biocides

With respect to the performance of exposure estimations for biocidal active substances, significant progress has been made in recent years in relation to the release or revision of guidance documents. The comprehensive guidances, opinions, or recommendations available are intended to assist applicants in the identification of exposure models most appropriate for their intended applications. These exposure models are largely based on assumptions with respect to exposure durations and indicative exposure values which were compiled in the framework of surveys performed under real application conditions. The following documents are generally used and accepted by regulatory authorities in the exposure assessment of biocidal active substances/biocidal products with respect to the identification of exposure models for specific applications and application types:

- User Guidance – Version 1 (TNsG 2002)
- Technical Notes For Guidance 2007 (TNsG 2007)
- Opinions of the Human Exposure Expert Group (HEEG)
- Recommendations of the Ad hoc Working Group on Human Exposure (HEAdhoc)
- Biocides Human Health Exposure Methodology Document (BHHEM 2015)
- DRAFT Guidance on Estimating Dietary Risk from Transfer of Biocidal Active Substances into Foods – Nonprofessional Uses
- BPR Guidance Volume III Human Health Parts B+C (Guidance on the Biocidal Products Regulation 2017)

In some instances, however, an acceptable exposure cannot be demonstrated on the basis of exposure modeling even if all feasible exposure refinement options have been exhausted. In these situations, the applicant needs to decide on whether to withdraw the concerned use or to perform exposure measurements under representative application conditions. The latter option is very demanding and requires a high degree of expertise including an intensive preparation in relation to planning and design as well as documentation and evaluation of the results of the operator exposure study.

Substances of Concern and Cumulative Risk Assessment

In the biocides area, the exposure and risk assessment process is not restricted to the active substance(s) only but needs also to consider "Substances of Concern" (SoCs). SoCs are understood as nonactive substances in a biocidal product which due to their intrinsic hazardous properties trigger or contribute to the classification of a biocidal product or substances which have been assigned a "IOELV" (Indicative Occupational Exposure Limit Value or Community workplace exposure limits). The latter type of substances requires a quantitative risk

characterization for local effects via the inhalation route in the intended applications. A further group of SoCs comprises substances which are active substances on their own but which have no biocidal function in the concerned biocidal product but rather function as a coformulant such as solvents. For this kind of SoCs, a quantitative risk characterization for systemic and/or local effects will be required in the biocidal product authorization process. Moreover, depending on the toxicological properties of both the active substance and the SoCs, the performance of a cumulative risk assessment may become necessary if common target organs/tissues or modes of action are identified in the respective toxicological data packages.

If the performance of a cumulative risk assessment will be needed, the overall risk, expressed as the "Hazard Index" (HI), is determined. This is accomplished by adding up the individual "Hazard Quotients" (HQ); the latter of which is formed by calculating the ratio of the estimated exposure and the respective reference dose (RfD) for the concerned substance with a view to the demonstration of acceptable risks in the intended biocidal application(s):

$$\begin{aligned} \mathrm{HI} = {} & \mathrm{HQ_A}(\mathrm{Exposure_A}/\mathrm{RfD_A}) + \mathrm{HQ_B}(\mathrm{Exposure_B}/\mathrm{RfD_B}) \\ & + \mathrm{HQ_C}(\mathrm{Exposure_C}/\mathrm{RfD_C}) + \cdots \end{aligned}$$

Regardless of the substances concerned in the overall exposure and risk assessment process, the following outcomes will be obtained:

- HI $<$ 1 (exposure $<$ AEL/AEC): safe use and no unacceptable health risk identified
- HI $>$ 1 (exposure $>$ AEL/AEC): no safe use, i.e., identified health risk not acceptable

In the latter case, a refinement of the risk characterization process is triggered with a focus on the following:

(a) Revisit of the toxicological data package with a view to increasing the reference values
(b) Refinement of the exposure assessment by taking into considerations application-specific information on in-use concentrations, application duration, and frequency including considerations on the conduct of use-specific operator exposure (OPEX) studies

Cross-References

► Adverse Effects Versus Non-adverse Effects in Toxicology
► Aims and Mission of Regulatory Toxicology
► Exposure Scenarios in Toxicology
► Limit Value Setting in Different Regulatory Areas of Toxicology

- ▶ Limit Values and Guideline Values in Regulatory Toxicology
- ▶ Read-Across Methodology in Toxicological Risk Assessment
- ▶ Registration and Approval in Regulatory Toxicology
- ▶ Risk Characterization in Regulatory Toxicology
- ▶ Single Substances Versus Combined Effects: Problems in Mixture Toxicology
- ▶ The Regulatory Process in Toxicology
- ▶ Toxicological Risk Assessment
- ▶ Working Areas of Regulatory Toxicology

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Part VIII

Addendum: Tables and Lists

Checklist: Toxicological Risk Assessment in Practice

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Michael Schwenk and H. Paul A. Illing

Contents

Abstract

The checklist gives brief practical hints for all those who are occasionally or professionally involved in toxicological risk assessment, risk management, and risk regulation, for example: when dealing with a new substance; a chemical incident; a pollution situation, where the full identity of the substances is unknown initially; a situation where human exposure exceeds guideline levels. It names typical questions arising in such situations, concerning the toxicological profile of the chemical, exposure levels, and risk. The checklist should be helpful in finding adequate solutions for many kinds of scenarios.

Keywords

Exposure assessment · Hazard identification · Internal exposure · Risk management · Safety factor · Toxic effect · Threshold · Toxicodynamics · Toxicokinetics · Vulnerable individuals

M. Schwenk (✉)
Tübingen, Germany

Formerly: Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

H. P. A. Illing
Formerly: Centre for Occupational and Environmental Health, University of Manchester, Manchester, UK

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Introduction

Each industrial chemical and pharmaceutical product has its specific hazard and risk profile that has been characterized in the premarket period using harmonized test methods and guidelines. Once on the market, the safety data sheet (for chemicals) and the package leaflets (for pharmaceuticals) provide information for safe and correct handling. When incidents nevertheless happen, all relevant information must be collected as base for risk assessment, without any delay.

Toxicological considerations in the development phase of a chemical/pharmaceutical resemble those, made in chemical emergencies. They include technical aspects like physicochemical features, toxic profile, exposure route, and risk estimation but also aspects of risk communication and risk management.

The checklist below deals with some key questions to be asked in specific situations. The left column depicts items that should be considered, when dealing with such questions. The right column (comments) provides some additional explanatory remarks.

Checklist and Comments

Checklist	Comments
Which are the steps of the risk regulation process?	
The WHO-IPCS document (IPCS 1994) identifies these as: **Risk assessment** (in four steps): Hazard identification Hazard characterization (including dose–response relationship) Exposure assessment Risk characterization **Risk management** Risk evaluation Emission and exposure control Risk monitoring	Risk assessments are made on the basis of a scientific examination of toxicity and exposure, leading to a risk characterization. The risk management process is aimed at developing an appropriate response (regulatory, technical, legal) to the hazard. Risk (or risk-benefit) evaluation, the first step in risk management, establishes a qualitative or quantitative relationship between risks and benefits of exposure to an agent and the influence of possible control measures on that evaluation. It may be necessary to examine relative risk and benefit for different agents used for the same purpose.
What data on toxic properties are needed for risk assessment?	
Chemistry Basic physical and chemical properties. Structure–activity relationships (if available) for the test substance and related substances. Chemical analysis	By proper assessment of the physicochemical properties (e.g., fat solubility, volatility, size of nanoparticles), it is often possible to get a first estimate of the risk level. This should soon be verified by analytical procedures.
Identification of toxic effects Animal testing results (acute, subacute, and chronic toxicity; carcinogenicity; and toxicity to reproduction) Evidence of irritation and sensitization Genotoxicity	Data quality (this includes whether appropriate protocols and audit procedures were employed) must be considered. For chemical assessment, Klimisch gradings are often used (Klimisch et al. 1997). The overall picture will emerge only from the

(continued)

Checklist	Comments
Results from in vitro tests Molecular mechanism of action Experience in humans (case reports, epidemiological studies, and experimental studies)	sum of all available information and will need reevaluation at periodic intervals and when new control measures are implemented. If in doubt, additional information/studies must be asked from poison control centers, manufacturers, and others.
Toxicodynamics Targeted organs, biomolecules Type of cell damage and repair Dose–response relationships (size of response). Rates of development and duration of effects.	Reversible or irreversible effect? Additive effects of mixtures (e.g., neurotoxicity)?
Toxicokinetics Absorption rates (oral, inhalation, dermal) Distribution Half-life Metabolites Routes and rates of elimination Experience with humans	Toxicokinetic data are often ignored in risk assessments – Which is a fault. Toxicokinetics determine levels and duration of organ exposure.
What information is provided by the dose–response relationship?	
Dose–response diagram Shows threshold above which effects can be observed (NOAEL/LOAEL/BMD). If the dose–response relationship is very steep, there is a very small safety margin. Shape of the curve influences values obtained by extrapolation to low doses (e.g., unit risk).	Non-sigmoidal dose–response relationship increases the uncertainty in extrapolation to low concentrations. NOAEL values of different studies often differ as they are the dose below the dose at which effects were seen and therefore depend on the dose intervals between doses in the study. They also depend on what parameters were measured in the studies. If in doubt, it should be checked as to whether one of the studies is better suited for a particular risk assessment.
How is an exposure assessment made?	
Source and external exposure	
Measurement or estimation of the extent of external exposure (in the intake, in the medium [air, water, food basket], or, using more complicated models, in the input to the medium [e.g., water] from the source [e.g., outlet sewer of chemical factory/sewage treatment works]). Observe all routes of exposure (oral, inhalation, dermal). Consider sensitive persons.	Exposure estimates can be extremely uncertain. Scenarios (models) should be clearly set out and estimates calculated according to standardized procedures. Estimates should not contain multiple "worst-case" assumptions (if the *P* value of 0.1 [i.e., 1 in 10 will show the effect] is applied three times, this gives a *P* value of 0.001 [1 in 1000]). Monte Carlo analysis is essential in these circumstances.
Internal exposure	
Calculation of the assumed maximum uptake on the basis of (worst-case) scenarios. Probabilistic assessment of the different routes of intake. Measurement of the internal concentration (human biomonitoring).	Human biomonitoring is the method of choice for internal exposure assessment – If available. It analyzes the substance in human body fluids of exposed versus unexposed (control) groups.

(continued)

Checklist	Comments
Which safety factors are often used?	
Usual safety factor for extrapolation for a threshold effect from a good animal data to a general human population = 100 (depends on circumstances). Some regulatory agencies use safety factors up to 10,000 (see, e.g., IPCS 1994).	Depending on the size of the selected safety factors, risk assessments can vary enormously even when the experimental database is identical. This can easily lead to dispute.
Why does epidemiology rarely find a threshold value?	
Uncertainty in the estimation of exposure of the effects at low doses **Variability** for example, due to polymorphism and age	Lack of thresholds in epidemiological studies may be artificially caused by the multiplication of several uncertainty factors plus interindividual variability of toxicodynamics and/or toxicokinetics.
Who belong to the vulnerable groups?	
Pregnant women (organogenesis of the embryo), infants, and children (organ development, toxicokinetics) Elderly and sick people (low functional reserves, low repair capacity) Allergic people (hypersensitivity)	Often, sensitive groups are given special regulatory protection in various laws (occupational safety, baby food, allergens, etc.). This must be considered in the risk management process.
What else must be considered in risk management?	
Protection philosophy for the population at risk (nature and extent of population covered, use of personal protective equipment, etc.) Guideline values and their rationale. Are they applicable? Verification of measurement results Quality assurance of the process	The safety philosophy may be for good hygiene practice, precautionary, or danger-oriented. It may be for a fit, working population or include vulnerable groups. In order that a risk assessment finds acceptance, it is important to understand the origin of existing regulations as well as the present state of scientific interpretation of the toxicological data.
What does "traffic light principle" mean in regulation?	
Green: No effect and no action required **Yellow**: Slightly below threshold level. Adequate action: Monitoring **Red**: Above the threshold of action. Swift action to reduce exposure and/or institute suitable control measures	Multistage systems such as the traffic light system are more flexible. Where only a single limit value exists, a brief or minor overrun may cause action or legal consequences, even if the excess is toxicologically irrelevant
When is a disease due to toxic substances?	
Causality can be assumed if exposure levels and exposure duration were sufficient and the response spectrum (the affected organ, expression) characteristic for a compound. The rarer the symptoms occur in daily life, the more secure a causal relationship can be assumed. Criteria to be considered are given in Hill	The causality principle is often presumed for toxic substances. But it is not easy to prove causality. With many drugs, possible unwanted effects are often overlooked. And the dramatic health effects of smoking and alcohol are often socially trivialized and ignored. Some dangerous substances produce very

(continued)

Checklist	Comments
(1965) and are applicable to all toxicological data, not only epidemiological data	specific disease patterns (e.g., asbestos and mesothelioma).
In which way can the modes of thinking influence the risk awareness?	
Scientific way of thinking ("objective risk") Risk assessment Risk comparison Risk management (technical)	Many social groups (toxicologists, engineers, politicians, stakeholders, arbitrator, government representatives, etc.) are potentially involved in risk communication and risk management.
Emotional way of thinking, e.g., by the general public (perceived risk) Risk acceptance or nonacceptance	In this process, it often happens that different ways of thinking collide. This leads to inner discomfort and confrontation. Knowledge of the various ways of thinking of the general public, as described by psychologists and sociologists, can reduce conflict.
Political way of thinking (perceived risk) Risk exaggeration (phantom risk) Risk trivializing Conclusion: Understanding the sociological and psychological aspects of risk perception and communication is critical to effective risk management.	A good moderator can help overcome these hurdles. Note: The eloquent charlatan and the lobbyist usually receive more credibility than the highly educated toxicologist and the regulator.
How to deal with knowledge gaps?	
During development of chemicals and pharmaceuticals Perform missing studies according to the needs and guidelines.	Check the current guidelines (guidelines are often updated); get information on the current state of expert discussions, notably in novel fields like biopharmaceuticals and nanoparticles.
In chemical emergencies such as uncontrolled emissions, spills, and accidents Protect humans and the environment, as adequate. Consider use of personal protection clothing. Organize medical assessment of exposed persons and treatment of injured individuals. Read safety data sheet. Contact regional poison center. Involve analytical laboratory. Check toxicological literature and current guidelines. Design an information sheet for the public. Advise the risk managing committee. Take measures for risk reduction and sanitation. Arrange follow-up study with measurement of exposure levels and disease parameters, and – Where possible – Measurement of the internal load of exposed vs unexposed persons.	Police, fire department, and medical first aid are the first to get alarmed; they organize initial rescue steps. Toxicologists may get involved early as well. They should then have knowledge about their responsibilities in the team. It may be favorable to involve additional experts who have experience with this kind of scenario. Rapid action and at the same time sensible dealing with uncertainties can be challenging. In cases where the identity of the contaminating/intoxicating substance is not clear, investigations should be made to reconstruct the cause of the incident and/or find the contamination source. Early support by an experienced trace-analysis laboratory is required. Repeated sampling and measurements allow to detect whether the chemical persists.

Cross-References

- ▶ Purpose and Methods of Risk Management in Regulatory Toxicology
- ▶ Risk Management in Toxicological Disasters
- ▶ The Regulatory Process in Toxicology
- ▶ Toxicological Risk Assessment

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Limit Values and Guideline Values in Regulatory Toxicology

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Karin Heine and Alexander Eckhardt

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K. Heine (✉)
FoBiG – Forschungs- und Beratungsinstitut Gefahrstoffe GmbH, Freiburg, Germany
e-mail: karin.heine@fobig.de

A. Eckhardt
Toxicology of Drinking Water and Swimming Pool Water, German Environment Agency, Bad Elster, Germany
e-mail: Alexander.Eckhardt@uba.de

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Abstract

Depending on the matrix (e.g., water, air) and the classification of a substance (e.g., contaminant), a multitude of limits and guidelines has been defined. The motivation for defining such a limit or guideline can be different. The most important reason is to protect the population from adverse health effects such as acute chronic toxicity or cancer. Another reason could be the protection of ecosystems which could be more vulnerable than humans. Moreover, aesthetic considerations, like the taste and/or odor of drinking water, can result in limitations of chemicals. In the following chapter, definitions of and examples for limits in water, air, or occupational environments are given. These lists are by no means exhaustible.

Introduction

Reference doses are important points of departure (POD) for calculating acceptable concentrations of contaminants in different media, e.g., food, water, or air. Since they are given on a per kg bodyweight base, sensitive population groups such as infants can be taken into account when necessary.

Reference Doses

Acceptable Daily Intake (*ADI*) Values

The World Health Organization (WHO) defined Acceptable Daily Intake (ADI) values for pesticides, food additives, and veterinary pharmaceuticals. The ADI values form the basis for the maximum concentration of contaminations in food. These values can be considered safe, even if a lifetime exposure is assumed (see Table 1 or references mentioned "Resources").

Acute Reference Dose (*Acute RfD*)

In order to avoid health risks caused by a single exposure exceeding the ADI, the WHO has established acute reference doses (acute RfD; see Table 1) for some pesticides. This is supposed to help local authorities to deal with spills or accidents.

Contaminants

If the uptake of a contaminant is per definition of the WHO acceptable, because the contaminant is useful, these contaminations become "tolerable." Early on (since 1972), provisional tolerable weekly intake (PTWI) values were defined for metals,

Table 1 Acceptable Daily Intake (*ADI*) values and acute reference doses (*RfD*) of selected pesticides and the year of their last evaluation (http://apps.who.int/pesticide-residues-jmpr-database)

Substance	ADI mg/kg/d (last evaluation)	Acute RfD mg/kg/d (last evaluation)
Abamectin	0.0025 (2008)	
Aldicarb	0.003 (1992)	0.003 (1995)
Amitrole	0.002 (1997)	
Azinphos-methyl	0.03 (2007)	0.1 (2007)
Benomyl	0.1 (1995)	
Carbaryl	0.008 (2001)	0.2 (2001)
Carbofuran	0.001 (2008)	0.001 (2008)
Chlorpyrifos	0.01 (2005)	0.1 (2004)
Chlorpyrifos-methyl	0.01 (2009)	0.1 (2009)
Cyfluthrin	0.04 (2006)	0.04 (2006)[a]
Cyhexatin	0.003 (2005)[b]	0.02 (2005)[c]
Cypermethrin	0.02 (2006)	0.04 (2006)[d]
2,4-D	0.01 (2001)	n.l.c.n.[e]
Deltamethrin	0.01 (2009)	0.05 (2000)
Diazinon	0.005 (2006)	0.03 (2001)
Dichlorvos	0.004 (1993)	0.1 (2012)
Dicofol	0.002 (1992)	0.2
Dimethoate	0.002 (2006)	0.02 (2003)[f]
Diphenylamine	0.08 (1998)	n.l.c.n.[e]
Diquat	0.006 (2013)	0.8 (2013)
Endosulfan	0.006 (1998)	0.02 (1998)
Ethylene thiourea	0.004 (1993)	
Fenamiphos	0.0008 (1997)	0.003 (2002)
Fenvalerate	0.02 (2012)	0.2 (2012)
Ferbam	0.003 (1996)[g]	
Lindane	0.005 (2002)	0.06 (2002)
Malathion	0.3 (1997)	2.0 (2003)
Mancozeb	0.03 (1993)[h]	
Maneb	0.03 (1993)[h]	
Methamidophos	0.004 (2002)	0.01 (2002)
Methomyl	0.02 (2001)	0.02 (2001)
Metiram	0.03 (1993)[h]	
Paraquat	0.005 (2003)	0.006 (2003)
Parathion	0.004 (1995)	0.01 (1995)
Parathion-methyl	0.003 (1995)	0.03 (1995)
Permethrin	0.05 (1999)	1.5 (2002)
Propineb	0.007 (2004)	0.1[i] (2004)
Propoxur	0.02 (1989)	
Pyrethrin	0.04 (2008)	0.2 (2003)

(continued)

Table 1 (continued)

Substance	ADI mg/kg/d (last evaluation)	Acute RfD mg/kg/d (last evaluation)
2,4,5-Trichlorophenoxyacetic acid	0.03 (1981)	
Thiabendazole	0.1 (2019)	1 (2019)[j]
Thiophanate-methyl	0.09 (2017)	1 (2017)
Thiram	0.01 (2003)	
Zineb	0.03 (1993)[h]	
Ziram	0.003 (1996)[g]	

[a]Group acute RfD with beta-cyfluthrin
[b]Group ADI with azocyclotin
[c]For women of childbearing age
[d]Group acute RfD with alpha- and zeta-cypermethrin
[e]No limit considered necessary
[f]Sum of dimethoate and omethoate
[g]Group ADI for ferbam and ziram
[h]Group ADI for mancozeb, maneb, metiram, and zineb
[i]Interim ARfD
[j]0.3 mg/kg bw for women of childbearing age

which accumulate in the human body due to their ubiquity. The definition of a tolerable weekly intake is motivated by the fact that one increased intake per week caused, for example, by certain foods is irrelevant. An example for this can be the uptake of methylmercury via fish. Later on, this concept was extended to substances (e.g., mycotoxins) that are not accumulating in the human body or that can diffuse into foods from packaging materials. In some cases, the limit was referred to a daily intake (PMTDI = provisional maximum tolerable daily intake). For pesticides which are banned but still contaminants of food products, the original ADI value was transferred into a PTDI value (provisional tolerable daily intake). MTDI (maximum tolerable daily intake) values were derived for substances that are essential for human nutrition but for which the range between physiological needs and toxic dose is very small, e.g., copper or iron. *TDI* (tolerable daily intake) values are the basis of the WHO's drinking water guidelines. Table 2 depicts tolerable intake values of some chemicals.

Tolerable Absorbed Doses (*TRD*)

For chemicals that are also important contaminants in soil, the so-called tolerable absorbed doses ("tolerierbare resorbierte Dosis," TRD) were derived by the Research and Advisory Institute for Hazardous Substances ("Forschungs- und Beratungsinstitut Gefahrstoffe," FoBiG) based in Freiburg, Germany. The values given in Table 3 are calculated for safe, lifetime exposure, either after oral or

Table 2 Tolerable intake values of selected chemicals and the year of their last evaluation. (Data is available from FAO (2019) and WHO (2012, 2017, 2019))

Substance	Type of limit	Amount [mg/kg/d] or [*mg/kg/week*]	Last evaluation
Acrylonitrile		n.s.l.r[a]	2000
Aflatoxins		ALARA[b]	1998
Aldrin (sum of aldrin + dieldrin)	PTDI[c]	0.0001	1994
Aluminum	PTWI[d]	*1*	2007
Arsenic	PTDI	0.0003	2011
Cadmium	PTWI	*0.007*	2000
Chloral hydrate	TDI	0.0045	2005
Chlorine	TDI[e]	0.15	1993
Chlorobenzene	TDI	0.0035	2003
Copper	MTDI[f]	10	2003
Cyanide, free	TDI	0.045	2017
2,4-DB	TDI	0.03	1993
DDT	PTDI	0.01	2000
o-Dichlorobenzene	TDI	429	1993
p-Dichlorobenzene	TDI	107	1993
1,1-Dichloroethene	TDI	0.046	2005
trans-1,2-Dichloroethene	TDI	0.017	2003
1,2-Dichloropropane	TDI	0.014	2003
Di(2-ethylhexyl)adipate	TDI	0.28	2003
Di(2-ethylhexyl)phthalate	TDI	0.025	1993
Dichloromethane	TDI	0.006	1993
Dichlorprop	TDI	0.0364	1993
Dieldrin	PTDI	0.0001	1994
Epichlorohydrin	TDI	0.00014	2003
Ethylbenzene	TDI	0.0971	2003
Formaldehyde	TDI	0.15	1993
Heptachlor	PTDI	0.0001	1994
Heptachlor epoxide	PTDI	0.0001	1994
Hexachlorobutadiene	TDI	0.0002	2003
Iodine	PMTDI[g]	0.017	1988
Iron	PMTDI	0.8	1983
Lead		n.s.l.r.	2016
MCPA	TDI	0.0001	2012
Mercury	PTWI	0.002	2004
Methylmercury	PTWI	*0.0033*	1998
Ochratoxin A	PTWI	*0.0001*	2007
Patulin	PMTDI	*0.0004*	1995
Pentachlorophenol	TDI	0.003	1998
Polychlorinated dibenzodioxins/-furans + PCB	PTMI[h]	70 pg/kg/month	2001

(continued)

Table 2 (continued)

Substance	Type of limit	Amount [mg/kg/d] or [*mg/kg/week*]	Last evaluation
Styrene	TDI	0.0077	1993
Tetrachloroethene	TDI	0.014	1993
Tetrachloromethane	TDI	0.0014	2003
Tin (inorganic)	TWI	*14*	2005
Toluene	TDI	0.223	2003
2,4,5-TP (fenoprop)	TDI	0.003	1993
Tributyltin oxide	TDI	0.00025	2018
Trichloroacetic acid	TDI	0.0325	2003
1,2,4-Trichlorobenzenes[i]	TDI	0.0077	2003
1,1,1-Trichloroethane	TDI	0.6	2003
Trichloroethene	TDI	0.00146	2005
Trifluralin	TDI	0.0075	1993
Xylenes	TDI	0.179	1993
Zinc	PMTDI	1	1982

[a]No safe level recommended
[b]As low as reasonably achievable
[c]Provisional tolerable daily intake
[d]Provisional tolerable weekly intake
[e]Tolerable daily intake
[f]Maximum tolerable daily intake
[g]Provisional maximum tolerable daily intake
[h]Provisional tolerable monthly intake
[i]All isoforms

respiratory intake. In order to compare those values with other limits like ADI, the absorption rate has to be taken into account.

Reference Dose and Reference Concentration (RfD, RfC)

The US Environmental Protection Agency (US-EPA) has established dose limits for oral (reference dose, RfD) or respiratory (reference concentration, RfC) uptake of many chemicals. Based on these values, the US limits for air, water, and food were defined. Listing all of these would exceed the scope of the book. The list can be found on the Internet at http://www.epa.gov/iris.

Occupational Safety and Health

According to the World Health Organization (see WHO in "Resources"), "occupational health deals with all aspects of health and safety in the workplace and has a strong focus on primary prevention of hazards. The health of the workers

Table 3 Tolerable resorbed dose (*TRD*) of selected chemicals from Eikmann et al. (2010)

	Oral intake		Respiratory intake	
Substance	[µg/kg/d]	Absorption [%]	[µg/kg/d] | [µg/m^3]	Absorption [%]
Aldrin	0.08	100	0.1 | 0.7 (P)	50
Antimony	0.07 (P)	20	– | 0.08	
Arsenic	0.8	100	1 | 50	30
Benzene	10	100	7 | 50	50
Beryllium	0.015 (P)	1		
Cadmium	0.025 (P)	5	0.035 (P) | –	
Chlorobenzene	70	100	60 | 400	50
Chromium (VI)	5		0.014 | 0.050	
Copper	25 (P)	50		
Cyclohexane			400 | 5600	25
Cyanides	10	100	10 | 50 (gas) 70 (particle)	70
DDT	1 (P)	100		
o-Dichlorobenzene	900	100	500 | 2,900	60
p-Dichlorobenzene	300	100	300 | 1,800	60
1,2-Dichloroethane	190	100	200 | 5,600	75
Dichloromethane	60 (P)	100	150 | 1,000	60
2,4-Dichlorophenol	9	100		
1,2-Dichloropropane	25 (P)	100		
Di-2-(ethylhexyl)-phthalate (DEHP)	30	60		
1,3-Dinitrobenzene	1	100	1 | 7	50
Diphenylamine	20	100		
Ethylbenzene	300 (P)	100	700 | 5,000	50
Hexachlorobenzene	0.030	100		
α-Hexachlorocyclohexane	0.100	100	0.025 | 0.088	100
β-Hexachlorocyclohexane	0.020	100	0.005 | 0.02 (P)	100
γ-Hexachlorocyclohexane	0.330	100	0.080 | 0.3	100
Hexachlorocyclohexane, mixture[a]	0.020	100	0.005 | 0.02	100
n-Hexane			100 | –	20
Lead	1 (P)	50 (children)	1 | 9 (P)	40
Mercury, inorg.	0.015	7	0.030 | 130	80
Mercury, org.	0.05	100		
Nickel	0.08	6	– | 0.170	
Nitrobenzene	2 (P)	100	2 | 7	100
2-Nitrotoluene	45	100		
3-Nitrotoluene	85	100		
4-Nitrotoluene	15	100		
N-Methyl- *N*-2,4,6-tetranitroaniline	15	100	15 | 100	50
PCB	0.015	100		

(continued)

Table 3 (continued)

Substance	Oral intake		Respiratory intake	
	[µg/kg/d]	Absorption [%]	[µg/kg/d] | [µg/m^3]	Absorption [%]
PCDD/F	0.000 001			
N-Nonane			500 | 5,800	30
Pentaerythritol tetranitrate (PETN)	3	100	3 | 21	50
Phenol			15 | 50	100
Styrene	260	100	260 | 100	70
Tetrachloroethene	20 (P)	100	30 | 200 (P)	50
Toluene	200 (P)	100	100 | 700	50
Trichloromethane	10 (P)	100		
1,2,4-Trinitrobenzene	15 (P)	100	5 | 18 (P)	100
1,1,1-Trichloroethane	500 (P)	100	500 | 5,800	30
Trichloroethene			80 | 560 (P)	50
1,3,5-Trimethylbenzene, all isoforms			100 (P) | 600	60
2,4,6-Trinitrotoluene	0.5	100	0.5 | 2.3	75
Vanadium	0.150	3	– | 1 (V_2O_5)	
Vinyl chloride	2	100	2 | 18	40
Xylenes	150	100	200 | 1,000	65

P provisional, due to large uncertainties

[a] $\left(\sum\left(\frac{\alpha HCH}{5}+\beta HCH+\frac{\gamma HCH}{16}\right)\right)$

has several determinants, including risk factors at the workplace leading to cancers, accidents, musculoskeletal diseases, respiratory diseases, hearing loss, circulatory diseases, stress related disorders and communicable diseases and others." Therefore, the European Union (EU) established the Occupational Safety and Health Agency to protect workers from occupational health hazards. Its abbreviation is "EU-OSHA" to be distinguishable from its sister agency in the USA, which is referred to as "OSHA." Every country of the EU and the EFTA (European Free Trade Association) has its own so-called focal points, which make up the national partners of EU-OSHA. Five priority groups were formed to satisfy the special needs of some of the most vulnerable workers: young workers, women, people with disabilities, migrant workers, and aging workers. To facilitate the implementation of EU directives on a national basis, the EU-OSHA passed several guidelines for topics such as workplaces, personal equipment, chemical agents, or physical hazards (see "Resources"). In Germany, the Federal Agency for Occupational Safety and Medicine (AGS 2020) has passed several technical guidelines (TRGS 900, 903, and 905) to implement the directive (EG) Nr. 1272/2008 (CLP) on classification, packaging, and labeling of dangerous substances. The guideline TRGS 900, for example, regulates the limits of

Table 4 Workplace limits of selected chemicals in Germany (TRGS 900) and the USA (OSHA)

	TRGS 900	US-OSHA
Substance	Concentration [mg/m^3]	Concentration [mg/m^3][a]
Sulfur dioxide (SO_2)	2.7	13
Carbon disulfide	30	63
Carbon monoxide	35	55
Dichloromethane	180	180[b]
Styrene	86	430
Tetrachloroethylene	69	690
Toluene	190	770

[a]Since most values provided by OSHA are given in ppm, transformation into mg/m^3 was based on a pressure of 1013 hPa and a temperature of 20 °C

[b]OSHA did not establish an exposure limit for dichloromethane. This value was established by the American Conference of Governmental Industrial Hygienists (ACGIH) and is quoted by OSHA

exposure against some 350 chemicals (AGS 2019). Similar regulations were passed in every member state of the EU, the USA (for link see "Resources"), and other countries. In all regulations, different aspects of toxicology, i.e., acute and chronic exposure or carcinogenic potential, are considered. From 1995 to 2018, the Scientific Committee on Occupational Exposure Limits (SCOEL) assisted the European Commission in assessing chemicals and their effects on workers on the European level. Since 2019, this task is fulfilled by the ECHA Risk Assessment Committee (RAC). Information about the activities of the RAC can be found on the web (for link see "Resources" – ECHA and RAC 2020). Currently, five opinions on occupational exposure limits (OEL) have been adopted, and another two are pending. Although the lists of substances that are regulated in Europe and the USA are quite similar, the limits can vary considerably as can be seen from Table 4.

Drinking Water

WHO Guidelines for Drinking Water Quality (WHO-GLDWQ)

The WHO recommends limits for organic as well as inorganic chemicals in its "Guidelines for Drinking-water Quality," which are not legally binding (see Table 5 or WHO 2017). Especially in countries without national regulations on drinking water, they can be used as guidelines. They are mostly derived from toxicological data, and the quality of the water bodies themselves is given only limited consideration. The European drinking water directive is currently revised, and new parameter values for lead, chromium, and perfluorinated chemicals will be implemented.

Table 5 Comparison between the WHO guidelines on drinking water quality and the limits in the German drinking water regulation (TrinkwV 2019)

Parameter	WHO [mg/l]	TrinkwV [mg/l]
Inorganic		
Aluminum		0.200
Ammonia		0.500
Antimony	0.020	0.0050
Arsenic	0.010[a]	0.010
Barium	0.7	
Beryllium	0.01	
Boron	0.5	1.0
Cadmium	0.0030	0.0030
Chloride		250
Chromium	0.050	0.050
Copper	2.0	2.0
Cyanide	0.07	0.050
Fluoride	1.5	1.5
Iron		0.2
Lead	0.010	0.010[b]
Manganese	0.4	0.050
Mercury	0.0010	0.0010
Nickel	0.020	0.020
Nitrate (as NO_3^-)	50	50
Nitrite (as NO_2^-)	0.20	0.50
Selenium	0.010	0.010
Sodium	200	200
Sulfate		250
Uranium	0.030	0.010
Organic		
Acrylamide	0.0005[c]	0.0001
Benzene	0.010[c]	0.0010
Tetrachloromethane	0.004	
1,2-Dichlorobenzene	1.0	
1,4-Dichlorobenzene	0.3	0.0030
1,2-Dichloroethane	0.030	
1,1-Dichloroethene	0.030	
1,2-Dichloroethene	0.050	
Di(2-ethylhexyl)adipate	n.l.c.n.	
Di(2-ethylhexyl)phthalate	0.008	
Dichloromethane	0.02	
EDTA	0.60	
Epichlorohydrin	0.00040	0.00010
Ethylbenzene	0.30	
Hexachlorobutadiene	0.0006	
Monochlorobenzene	n.l.c.n.	

(continued)

Table 5 (continued)

Parameter	WHO [mg/l]	TrinkwV [mg/l]
Nitrilotriacetic acid	0.20	
Polyaromatic hydrocarbons (PAH)	0.00070	0.00010[d]
Benzo[a]pyrene	0.0007[c]	0.000010
Styrene	0.020	0.010
Tetrachloroethene + trichloroethane	0.040	
Toluene	0.7	
Tributyltin oxide		
Trichlorobenzene (sum) 1,2,4-Trichlorobenzene	n.l.c.n.	
1,1,1-Trichloroethane	n.l.c.n.	
Vinyl chloride	0.00030[c]	0.00050
Xylenes	0.5	
Pesticides		
Alachlor	0.02	0.00010[e]
Aldicarb	0.01	0.00010
Aldrin/dieldrin	0.000030	0.000030
Atrazine	0.002	0.00010
Carbofuran	0.007	0.00010
Chlordane	0.0002	0.00010
Chlortoluron	0.03	0.00010
2,4-D	0.03	0.00010
2,4-DB	0.09	0.00010
DDT	0.001	0.00010
1,2-Dibromo-3-chloropropane	0.001	0.00010
Dichlorprop	0.1	0.00010
Fenoprop	0.009	0.000030
Heptachlor + heptachloroepoxide	n.l.c.n.	0.00010
Hexachlorobenzene	n.l.c.n.	0.00010
Lindane	0.002	0.00010
MCPA	0.002	0.00010
Mecoprop	0.01	0.00010
Methoxychlor	0.02	0.00010
Pentachlorophenol	0.009	0.00010
Permethrin	n.l.c.n.	0.00010
Propanil	t.m.	0.00010
2,4,5-T	0.009	0.00010
Disinfecting agents		
Chlorine	5.0	
Disinfection by-products		
2,4,6-Trichlorophenol	0.02	0.01
Bromate	0.01	
Chloral hydrate	0.01	
Chlorite	0.7	
Dibromoacetonitrile	0.07	

(continued)

Table 5 (continued)

Parameter	WHO [mg/l]	TrinkwV [mg/l]
Dichloroacetic acid	0.05	
Dichloroacetonitrile	0.02	
Formaldehyde	0.90	
Trihalomethanes	6[f]	0.050
Chloroform	0.20	
Bromoform	0.10	
Bromodichloromethane	0.060	
Dibromochloromethane	0.10	
Trichloroacetic acid	0.20	
Trichloroacetonitrile	i.d.	

n.l.c.n. no limit considered necessary; *t.m.* more toxic metabolites, but insufficient data on them; *i.d.* insufficient data

[a]Additional risk for skin cancer 10^{-4}

[b]Limit of 0.025 mg/l until 11/30/2013, 0.010 mg/l from 12/01/2013

[c]Additional risk for cancer 10^{-5}

[d]Sum of the following substances: benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, indeno[1,2,3-cd]pyrene

[e]General limit for pesticides in German drinking water, when no lower value is necessary, sum 0.5 µg/l

[f]For authorities wishing to establish a total THM standard to account for additive toxicity, the following fractionation approach could be taken: the sum the value of each THM divided by this guideline value has to be <1

German Regulation on Drinking Water

The German drinking water regulation ("Trinkwasserverordnung," TrinkwV) was ratified in February 2001 and entered into force on January 1, 2003. It was last modified on January 3, 2018. Most of the limits are based on the guidelines of WHO. Exceptions are the limits for pesticides. The WHO states a health-based limit for each pesticide, whereas in the EU and thus also in Germany, the drinking water limit of 0.1 µg/L is politically motivated. In addition, the EU has set a limit for the sum of all pesticides (i.e., 0.5 µg/l; see Table 5).

Air

World Health Organization (WHO) *Air Quality Guidelines*

Clean air is a basic need for human well-being and health. The World Health Organization (WHO) therefore first published its Air Quality Guidelines for Europe in 1987, and a second edition was issued in 2000 (WHO 2000). Within these guidelines, guideline values for various inorganic, organic, and so-called classical pollutants (i.e., nitrogen dioxide, ozone, particulate matter, and sulfur dioxide) for air outdoors were established. Moreover, guideline values for indoor air pollutants were

Table 6 Air quality guideline values as provided in the second air quality guidelines for Europe, the global update in 2005 and guidelines for indoor air quality: selected pollutants (WHO 2010)

Substance	Concentration [μg/m^3, if not otherwise indicated] or unit risk (UR)[a]	Averaging period
Classical pollutants		
Sulfur dioxide (SO_2)	20	24 h
	500	10 min
Nitrogen dioxide (NO_2)	40	Year
	200	1 h
PM_{10}[b]	20	Year
	50	24 h[c]
$PM_{2.5}$[b]	10	Year
	25	24 h[c]
Ozone[b]	100	Daily maximum 8-h mean
Organic pollutants		
Acrylonitrile	UR: 2×10^{-5} (lung)	
Benzene	UR: 6×10^{-6} (blood: leukemia)	
Carbon disulfide	100	24 h
Carbon monoxide	7 mg/m^3	24 h
	10 mg/m^3	8 h
	35 mg/m^3	1 h
	100 mg/m^3	15 min
1,2-Dichloroethane	700	24 h
Dichloromethane	450	1 week
	3,000	24 h
Formaldehyde	100	30 min
Polycyclic aromatic hydrocarbons[d]	UR: $8{,}75 \times 10^{-5}$ (lung)	
Styrene	260	1 week
Tetrachloroethylene	250	Year
Toluene	260	1 week
Trichloroethylene	UR: 4.3×10^{-7} (lung, testis)	
Vinyl chloride	UR: 1×10^{6} (lung and other sites)	
Inorganic pollutants		
Arsenic	UR: 1.5×10^{-3} (lung)	
Asbestos	At a concentration of 500 fibers[e]/m^3, the following ranges of lifetime risk estimates are made: 10^{-6} to 10^{-5} (lung cancer in a population where 30% are smokers) 10^{-5} to $10{-}^{4}$ (mesothelioma)	
Cadmium	0.005	Year
Chromium (VI)	UR: 4×10^{-2} (lung)	
Hydrogen sulfide	150	24 h
Lead	0.5	Year
Manganese	0.15	Year

(continued)

Table 6 (continued)

Substance	Concentration [μg/m^3, if not otherwise indicated] or unit risk (UR)[a]	Averaging period
Mercury	1	Year
Nickel	UR: 4×10^{-4} (lung)	
Platinum		
Refractory ceramic fibers	UR: 1×10^{-6} (fiber/l)$^{-1}$ (lung)	
Vanadium	1	24 h
Indoor air pollutants		
Environmental tobacco smoke	UR: approximately 1×10^{-3}	
Man-made vitreous fibers	See above (refractory ceramic fibers, for most other MMVF data are considered inadequate to establish AQG)	
Radon	Reference level: 100 Bq/m^3 (a)	Year
	300 Bq/m^3 (b)	Year
	UR[f]: 0.6×10^{-5} per Bq/m^3 (nonsmokers)	
	UR[f]: 15×10^{-5} per Bq/m^3 (smokers)	
Naphthalene	10	Year

[a]Unit risk: Cancer risk estimates for lifetime exposure to concentration of 1 μg/m^3
[b]Air quality guideline (AQG) values
[c]24-h concentration: 99th percentile (3 days/year)
[d]Based on benzo[a]pyrene
[e]Fibers measured by optical methods
[f]Excess lifetime risks (by the age of 75 years) for lung cancer of lifelong nonsmokers or current smokers (15–24 cigarettes/day)
1. To minimize health hazards due to indoor radon exposure
2. Applies only if the Reference Level mentioned in (a) cannot be reached under the common country-specific conditions

provided. In 2005, a global update became available for the classical pollutants (WHO 2006). There are also guidelines for emissions of gases and chemicals from furnishings and building materials that collect indoors (WHO 2010), and the most recent WHO guideline deals with indoor air quality – household fuel combustion (WHO 2014). In this guideline, no need for review of the evidence informing the published WHO guidelines for air quality (AQG) was identified. Currently available air quality guideline values are provided within Table 6.

Immission Values Based on EU Directives

Within the European Union, there are directives dealing with several air pollutants (e.g., nitrogen dioxide, particulate matter, lead, and sulfur dioxide), which provide immission limit values. They are mostly based on the recommendations of the WHO

Table 7 Immission limit values and target values for protection of human health taken from 39th BImSchVO, which represents national implementation of current European legislation

Substance	Concentration [µg/m^3]	Averaging period	Allowed frequency of excess per year
Sulfur dioxide (SO_2)	125	Day	3
	350	1 h	24
Nitrogen dioxide (NO_2)	40	Year	–
	200	1 h	18
PM_{10}	40	Year	–
	50	Day	35
$PM_{2.5}$[a, b]	25	Year	
Ozone[c]	120	Daily maximum 8-h mean	25
Lead	0.5	Year	
Benzene	5	Year	
Carbon monoxide (CO)	10,000	Daily maximum 8-h mean	
Arsenic[d]	6 ng/m^3	Year (total content in PM_{10} fraction)	
Cadmium[d]	5 ng/m^3		
Nickel[d]	20 ng/m^3		
Benzo[a]pyrene[d]	1 ng/m^3		

[a]Immission limit value, but starting from January 1, 2020, further reduction of $PM_{2.5}$ exposure according to national target value is foreseen. This target value depends on the value of the indicator for the average $PM_{2.5}$ exposure according to §15 in the reference year 2010. The assessment shall be carried out by the German Environment Agency in accordance with Annex 12, Section B

[b]This commitment was also included in the recast of the National Emission Ceilings Directive (NEC Directive) adopted in 2016 and supplemented by a target for 2030 (EC 2001, 2016). By that date, German $PM_{2.5}$ emissions must be reduced by 43% compared to 2005 (for link see "Resources")

[c]Target value, which will be the long-range target value without allowance of excess

[d]Target values which will be valid from January 1, 2013 onward

(see above) and are designed to protect human health from harmful environmental influences. In Germany, the respective legislation is based on the Federal Immission Control Act ("Bundes-Immissionsschutzgesetz," BImSchG, last updated in 2011). Limit values were defined earlier within the 22nd Regulation of Federal Immission Control (22. BImSchVO as of October 26, 1993). In 1996, the Framework Directive 96/62/EC entered into force and subsequently various sub-directives ("daughter directives" in Annex I of Framework Directive; 1999/30/EC; 2000/69/EC; 2002/3/EC; 2004/107/EC), in which immission limit values were described to ensure protection of human health, as well as protection of the environment (ecosystems and vegetation). For implementation of revisions made, the European Directive 2008/50/EC entered into force (May 21, 2008). Afterward in Germany, the 22nd amended BImSchVO was withdrawn and newly regulated on a national level via the 39th Regulation of Federal Immission Control (39. BImSchVO as of August 2, 2010; see Table 7 and for link see "Resources"). In case of carcinogenic substances (e.g., benzene), the limit values are set to an extra risk of $1{:}1 \times 10^{-6}$.

Table 8 Immission limit values for protection from hazard to human health according to "TA Luft"

Substance	Concentration [µg/m^3]	Averaging period	Allowed frequency of excess per year
Sulfur dioxide (SO_2)	50	Year	–
	125	24 h	3
	350	1 h	24
Nitrogen dioxide (NO_2)	40	Year	–
	200	1 h	18
PM_{10}	40	Year	–
	50	24 h	35
Lead[a]	0.5	Year	
Benzene	5	Year	
Tetrachloroethylene	10	Year	

[a]Lead and its inorganic compounds as part of particulate matter (PM_{10}), given as Pb

Technical Instruction on *Air Quality* Control ("TA Luft")

Based on the Federal Immission Control Act ("Bundes-Immissionsschutzgesetz," BImSchG, last updated in 2011), an administrative act was issued. This was the technical instruction on air quality control ("TA Luft" as of July 24, 2002, which replaces the "TA Luft" from 1986). Within this updated administrative act, emission and immission values are provided. They are used to control for construction and operation of industrial sites, which are subjects of approval. Emission values are precautionary values in order to protect against detrimental environmental influences. Immission values shall protect from hazard to human health (see Table 8) as well as from major disturbance or disadvantages.

Indoor Air Guide Values

Besides the WHO air quality guideline values indicated above (see Table 6), which can be used for indoor air as well, national committees are in place to establish guideline values for indoor air (see Table 9). In Germany, since 1993, there is a Committee on Indoor Guide Values ("Ausschuss für Innenraumrichtwerte," AIR, formerly "ad hoc working group"). AIR consists of technical experts from the Indoor Air Hygiene Commission (IRK) of the German Environment Agency and the Permanent Working Group of the Highest State Health Authorities ("Arbeitsgemeinschaft der Obersten Landesgesundheitsbehörden," AOLG). Until now, guide values for various organic compounds, hydrocarbons used as solvents, mercury vapors, and the inorganic gases carbon monoxide and nitrogen dioxide have been derived. There are two categories of guideline values: *RW-I* and *RW-II* (RW = "Richtwert" = guide value).

Table 9 Guide values of the last 5 years from AIR

Substance	RW-II[a] [µg/m^3]	RW-I[a] [µg/m^3]	Year of designation by AIR
Nitrogen dioxide (NO_2) (CAS no. 10102-44-0)	250 (60 min)	80 (60 min)	2018
2-Phenoxyethanol (CAS no. 122-99-6)	100	30	2018
Tetrachloroethene (CAS no. 127-18-4)	1,000	100	2017
Propan-1,2-diol (CAS no. 57-55-6)	600	60	2017
C7-C8-alkylbenzenes – total guide values: toluene, xylene, ethylbenzene	See comment b below		2016
Toluene (CAS no. 108-88-3)	3,000	300	2016
Formaldehyde (CAS no. 50-00-0)	Not derived	100	2016
Xylene total (CAS no. 95-47-6; 108-38-3; 106-42-3; 1330-20-7)	800	100	2015
2-Butanone oxime (CAS no. 96-29-7)	60	20	2015
2-Chloropropane (CAS no. 75-29-6)	8,000	800	2015

Further values are available, Resources: "Umweltbundesamt 2020" https://www.umweltbundesamt.de/themen/gesundheit/kommissionen-arbeitsgruppen/ausschuss-fuer-innenraumrichtwerte-vormals-ad-hoc

[a]Usually, these guidelines are given as long-term values. Averaging periods deviating from this are given in brackets, e.g., 60 min

[b]For each substance i, the ratio R_i: $R_i = c_i/RW_i$ is formed. In this, c_i is the concentration of the respective substance in indoor air and RW_i is the corresponding guide value. The respective R_i are then added to an R: $R = \Sigma R_i = \Sigma c_i/RW_i$. If R is exceeding the value of 1, the respective total reference value is exceeded. Applicable guide values:

Substance	RW-II a [µg/m^3]	RW-I a [µg/m^3]
Ethylbenzene	2000	200
Xylene total	800	100
Toluene	3000	300

RW-I is a precautionary guideline. It is anticipated that even with lifelong exposure below the RW-I concentration, no adverse effects on human health are to be expected. If the threshold is exceeded, additional and non-usual burden might be the case. If the measured concentration is between RW-I and RW-II, precautionary measures shall be taken (either by changing the consumer behavior or by technical or construction measures). RW-I guideline values can be used as target values for remediation purposes.

RW-II values are effect-related values, which are derived from current toxicological and epidemiological data on the respective substance taking assessment factors into account. Depending on the mode of action, the RW-II values might refer to short-term (RW-II-K) or long-term values (RW-II-L). In case that the RW-II value is reached or even exceeded, an acute need for action is indicated.

Food

Maximum Residual Levels (*MRLs*): International, European, and National Standards and Regulations

As fruits and vegetables are prone to various detrimental effects (e.g., diseases and pests), pesticides are used to ward off or at least minimize these negative consequences. On the other hand, it must be granted that plant protection products have no adverse effects on human health. Therefore, maximum residual levels (MRLs) are in place in order to prevent consumers from adverse health effects. Within Europe, maximum residue levels are defined as "upper levels of pesticide residues that are legally permissible in or on food or animal feed, based on good agricultural practice and the lowest consumer exposure necessary to protect vulnerable consumers." Most of the values are based on the ALARA principle (as low as reasonably achievable). If the MRL within one foodstuff is exceeded, trading might be forbidden, even if there is no adverse health effect yet.

The WHO and the FAO (Food and Agriculture Organization of the United Nations) jointly publish the *CODEX Alimentarius*, which provides international food standards for pesticides (also for veterinary drugs and food additives). The CODEX standards contribute to the safety, quality, and fairness of the international food trade. They are only recommendations but may often serve as a basis for national legislations. The standards are not listed here as they can easily be searched within the given Internet source (see "Resources").

In Europe, in 2005, a harmonized regulation was being introduced with Regulation (EC) No 396/2005 which entered into force in 2008. The text of the regulation is given on the homepage of the Federal Office of Consumer Protection and Food Safety, which includes the currently valid MRLs in Annex II, IIIA, and IIIB. As the list of regulated substances is quite extensive and would not fit into this chapter, we refer to the list as provided within the regulation. Moreover, an EU Pesticides Database was established in order to perform searches for MRLs (for link see "Resources"). In cases where no specific MRL was determined, a general level of 0.01 mg/kg has to be met.

Even though the European Regulation overrules the former, valid German Regulation on maximum residual levels ("Rückstands-Höchstmengenverordnung," RHmV, 1994, last updated in 2010), which was the national implementation of various former EU Directives (i.e., 90/642/EEC, 93/57/EEC, 93/58/EEC), this regulation still is in force concerning certain areas, for example, for safeners and synergists which are relevant co-formulants in plant protection products, or in case of category 11 (fish, fish products, shellfish, mollusks, and products of other freshwater or seawater fish). In case of group 12 (exclusive use as feeding stuff), still the German Regulation on Feeding stuff ("Futtermittelverordnung," FuttMV, 1981, last updated in 2018) is in place.

European Maximum Levels for Certain Contaminants in Foodstuffs

Contaminants are substances that have not intentionally been added to food. In general, they negatively influence the quality of food and may cause harm to

human health in certain cases. Basic principles are therefore regulated within Council Regulation 315/93/EEC, stating that food containing unacceptable amounts of contaminants based on considerations for the human health shall not be placed on the market, the level shall be kept as low as reasonably achievable (ALARA principle), and maximum levels must be set. These maximum levels are given in the Commission Regulation (EC) No 1881/2006, which entered into force in 2007 and was amended afterward several times. Currently, there are community measures for the following contaminants: mycotoxins (aflatoxins, ochratoxin A, *Fusarium* toxins, patulin, citrinin, ergot sclerotia, and ergot alkaloids), metals (cadmium, lead, mercury, inorganic tin), dioxins and PCBs, polycyclic aromatic hydrocarbons (PAH), 3-monochloropropanediol (3-MCPD) and glycidyl fatty acid ester, nitrates, and plant toxins (erucic acid, tropane alkaloids, and prussic acid). Even though only a small number of contaminants are regulated, the list is quite extensive as foodstuff description is detailed, and therefore here it is referred to the list included in the Commission Regulation (EC) No 1881/2006 and its amendments (for information please see Resources: European Commission).

Soil

Germany adopted a legislation to protect soil in 1998 (so-called Bundes-Bodenschutzgesetz, BBodSchG). Based on this legislation, the federal regulation on soil conservation and contaminated sites was adopted in 1999 ("Bundesbodenschutzverordnung," BBodSchV; see also "Resources"). Within this regulation, there are *precautionary values*, *trigger values*, and *action values* (i.e., "Vorsorge, Prüf- und Maßnahmenwerte"). These values are designed to apply for different routes of exposure (i.e., soil to humans, soil to plants, soil to groundwater). The values established for direct interaction (soil to humans) are the most relevant from the toxicological point of view (see Table 10). These values always consider different exposure scenarios (i.e., playground, residential area, park and recreation area, industrial sites) and are usually derived from the *TRD* values (tolerable absorbed dose; cf. paragraph on Tolerable Absorbed Doses (*TRD*)). In case of carcinogenic substances, an additional risk of $>5 \times 10^{-5}$ is assumed to be no more tolerable under defined exposure assumptions for trigger as well as action values.

When a trigger value is exceeded, a site-specific assessment has to be performed, in order to investigate if a detrimental effect to soil quality or even a hazardous waste site may be present. This site-specific determination allows the respective authority to decide if further action to reduce exposure is necessary.

In general, if action values specified for a certain exposure scenario (e.g., playground) are exceeded, it is assumed that detrimental effects to soil quality occurred or a hazardous waste site was identified, and an immediate need for action to reduce exposure exists. Action values are only available for polychlorinated dibenzodioxins or dibenzofurans.

Table 10 Trigger and action values for substances mentioned in Annex 2 of the "BBodSchV" (exposure pathway: soil to humans)

Trigger values [mg/kg dry weight]				
Substance	Playground	Residential area	Park and recreation area	Industrial site
Arsenic	25	50	125	140
Lead	200	400	1,000	2,000
Cadmium	10[a]	20[a]	50	60
Cyanides	50	50	50	100
Chromium	200	400	1,000	1,000
Nickel	70	140	350	900
Mercury	10	20	50	80
Aldrin	2	4	10	–
Benzo[a]pyrene	2	4	10	12
DDT	40	80	200	–
Hexachlorobenzene	4	8	20	200
Hexachlorocyclohexane (HCH mixtures or beta HCH)	5	10	25	400
Pentachlorophenol (PCP)	50	100	250	250
Polychlorinated biphenyls (PCB $_6$)[b]	0.4	0.8	2	40
Action values [ng l-TEQ/kg dry weight][c]				
Polychlorinated dibenzodioxins/ dibenzofurans (PCDD/F)	100	1,000	1,000	10,000

[a]In garden plots, which can be occupied by children and are used for growing food plants, a guidance value of 2 mg cadmium per kg dry weight has to be applied
[b]In case total amount of PCBs are determined, the measured values have to be divided by 5
[c]Total amount of 2,3,7,8-TCDD toxicity equivalents (according to NATO Committee on the Challenges of Modern Society)

A volume entitled "Calculation of guidance values for assessment of hazardous waste sites" is dedicated to the protection of soil and was published by the German Environment Agency (Bachmann et al. 2007). This supplementary volume includes the underlying calculations for all legally binding trigger values presented in Table 10. Moreover, references for further trigger values are given for approximately 50 additional substances, relevant for hazardous waste sites, especially abandoned military sites.

Body Burden

The Human Biomonitoring Commission is part of the German Environment Agency ("Umweltbundesamt," UBA). Members are recruited from federal and regional authorities, as well as from academia (universities, institutes of hygiene, medicinal clinics). The work of the commission was to establish various reference

Table 11 Reference values and Human Biomonitoring (*HBM*) values for various substances in urine or blood

	Reference values		Human biomonitoring (HBM) values		
Substance and matrix	Subpopulation	Reference value	Subpopulation	HBM-I	HBM-II
Lead (blood)	Children (girls 3–17 y, boys 11–17 y) Children (boys 3–10 y) Females (18-69 y) Males (18–69 y)	15 µg/l 20 µg/l 30 µg/l 40 µg/l	Children ≤12 years and females of reproductive age Other persons	Suspended	Suspended
Cadmium (urine)	Non-smoking - Children (3–14 years) - Adults (18–69 years)	0.2 µg/l 0.8 µg/l	Children and adolescents Adults	0.5 µg/l 1 µg/l	2 µg/l 4 µg/l
Mercury (urine)	Without amalgam fillings - Children (3–14 years) - Adults (18–69 years)	0.4 µg/l 1.0 µg/l	Children and adults	7 µg/l 5 µg/g Crea.	25 µg/l 20 µg/g Crea.
Mercury (blood)	Fish consumption ≤3 times/month - Children (3–14 years) - Adults (18–69 years)	0.8 µg/l 2.0 µg/l	Children and adults[a]	5 µg/l	15 µg/l
Thallium (urine)	Children (3–14 years) Adults (20–29 years)	0.6 µg/l 0.5 µg/l	General population	5 µg/l	/

(continued)

Table 11 (continued)

	Reference values		Human biomonitoring (HBM) values		
Substance and matrix	Subpopulation	Reference value	Subpopulation	HBM-I	HBM-II
Pentachlorophenol (serum)	Adults	12 µg/l	General population	40 µg/l	70 µg/l
Pentachlorophenol (urine)	Children (3–14 years) Adults (18–69 years)[b]	2.0 µg/l[c] 5 µg/l	General population	25 µg/l 20 µg/g Crea.	40 µg/l 30 µg/g Crea
∑ of the DEHP metabolites 5oxo- and 5OH-MEHP (urine)	Children (3–14 years) Adults (20–29 years)	280 µg/l 50 µg/l	Children (6–13 years) Women of childbearing age Males ≥14 years of age and remaining general population	500 µg/l 300 µg/l 750 µg/l	/ / /
∑ PCB (138 + 153 + 180) (serum x2)	See Table 12		Babies, small children, and women of childbearing age	3.5 µg/l	7 µg/l
Bisphenol A (urine)	Children (3–5 years) Children (6–14 years) Adults (20–29 years)	30 µg/l 15 µg/l 7 µg/l	Children Adults	100 µg/l 200 µg/l	/
Glycol ether which is metabolized to 2-methoxyacetic acid (MAA, urine)		/	General population	0.4 mg MAA/g Crea.	1.6 mg MAA/g Crea
Glycol ether which is metabolized to 2-ethoxyacetic acid (EAA, urine)		/	Adults	5 mg EAA/l	/
Σ DINCH®-metabolites OH-MINCH and cx-MINCH (urine)		/	Children Adults	3 mg/l 4.5 mg/l	/
DPHP-metabolites OH-MPHP and oxo-MPHP (urine)		/	Children Adults	1 mg/l 1.5 mg/l	/
Hexabromocyclododecane (HBCD(D))		/	General population	0.3 µg/g Fett (1.6 µg/l plasma)	/
Triclosan (urine)		/	Children Adults	2 mg/l 3 mg/l	/

2-Mercaptobenzo-thiazole (2-MBT, urine)[d]		/	Children Adults	4.5 mg/l 7 mg/l	/
Σ N-Methyl-2-pyrrolidone (NMP)-metabolites 5-hydroxy-NMP and 2-hydroxy-N-methylsuccinimide (urine)		/	Children Adults	10 mg/l 15 mg/l	30 mg/l 50 mg/l
Σ N-Ethyl-2-pyrrolidone (NEP)- metabolites 5-HNEP and 2-HESI (urine)		/	Children Adults	10 mg/l 15 mg/l	25 mg/l 40 mg/l
Σ 3-(4-Methyl-benzylidene)-camphor (4-MBC)-metabolites 3-4CBHC and 3-4CBC (urine)		/	Children Adults	0.3 mg/l 0.5 mg/l	/
PFOA (blood plasma)	Children (3–17 years) Women, men	3 µg/l 10 µg/l	General population Women of childbearing age	2 µg/l /	10 µg/l 5 µg/l
PFOS (blood plasma)	Children (3–17 years) Women Men	5 µg/l 20 µg/l 25 µg/l	General population Women of childbearing age	5 µg/l /	20 µg/l 10 µg/l
7-Hydroxy-citronellal, metabolite 7-hydroxycitronellyl acid (urine)[d]		/	Children Adults	9 mg/l 14 mg/l	/
DEHTP metabolite 5cx-MEPTP (urine)		/	Children Adults	1.8 mg/l 2.8 mg/l	/

Note: there are further reference values available for other substances (reference values are provided only in German; Resources: "Umweltbundesamt, Reference and HBM values")

Crea creatinine

[a]Derived from females in reproductive age. The use is recommended for other groups

[b]No reference value, but should there be analytical reliable and confirmed concentrations above the mentioned value, a special exposure must be expected

[c]Subpopulation only refers to adults who were living in homes where no wood preservatives had been used

[d]Possible sensitization not considered

Table 12 Reference values for persistent organic pollutants in whole blood [µg/l]

Age (years)	PCB 138	PCB 153	PCB 180	∑ PCB (138 + 153 + 180)	β-HCH	HCB	DDE Germany East	DDE Germany West
7–14	0.3	0.4	0.3	1.0	0.1	0.2	1.4	0.7
18–19	0.4	0.6	0.3	1.1	0.3	0.4	3	1.5
20–29	0.6	0.9	0.6	2.0	0.3	0.5	5	2
30–39	0.9	1.6	1.0	3.2	0.3	1.0	11	4
40–49	1.4	2.2	1.6	5.1	0.3	2.5	18	7
50–59	1.7	2.8	2.1	6.4	0.5	3.3	31	8
60–69	2.2	3.3	2.4	7.8	0.9	5.8	31	11

Note: Values for PCB 138, PCB 153, and PCB 180 and the sum of these PCBs are no longer listed on the homepage of the German Environment Agency ("Umweltbundesamt")

Table 13 Reference values for some polychlorinated biphenyls (*PCB*) and organochlorines in human breast milk [mg/kg fat]

Total DDT (applies only to women in western Germany)	Total PCB (138 + 153 + 180)[a]	β-HCH	HCB
0.5	0.5	0.07	0.06

[a]Total PCB = 1.64*(PCB 138 + PCB 153 + PCB180)

and Human Biomonitoring values for different toxic substances in various body fluids, which are further exemplified below (Tables 11, 12, and 13 and Resources "HBM-Commission").

The reference value describes the exposure of individuals or population groups compared to the ubiquitous background exposure (they are checked and updated continuously). The reference value for a specific chemical is established only on a statistical basis, after having collected a representative series of measurements (from a defined group of the general population). These values therefore have not been derived with respect to protecting human health.

In contrast to that, Human Biomonitoring (HBM)-I and HBM-II values are based on toxicological and epidemiological data. The derivation of HBM values relied on studies that allowed correlating the concentration of a substance or its metabolites in human body fluids and the occurrence of adverse effects. Such studies are lacking for most of the substances. Thus, since 2014, the HBM Commission also derives HBM values based on toxicologically justified tolerable daily intakes or other suitable parameters from animal experiments. Known uncertainties of this approach are weighed against urgently required HBM values for substances or their metabolites for which no suitable studies on health effects of low-dose environmental exposure are currently available.

According to the current opinion of the HBM committee, HBM-I values represent the concentration of a chemical in a defined biological material, below which no adverse health effect is expected and therefore no actions have to be taken. In case the *HBM-I value* is exceeded, but is still lower than the *HBM-II value*, further

measurements have to be performed, and the possible source of exposure should be identified. Moreover, the exposure to the source should be minimized. The HBM-I value thus represents a verification or control value.

HBM-II values represent the concentration above which a high possibility of an adverse health effect exists, thus resulting in acute need for action (i.e., reduction of exposure and biomedical care (advice)). The HBM-II value therefore represents an intervention or action value.

Cross-References

- ▶ Assessment of Limit Values in Regulatory Toxicology
- ▶ Limit Value Setting in Different Regulatory Areas of Toxicology
- ▶ Medical Aspects of Traffic Exhaust Regulations
- ▶ Regulation of Agrochemicals
- ▶ REACH and CLP: Its Role in Regulatory Toxicology
- ▶ Risk Assessment of Food Additives

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Appendix

Regulatory Toxicology: Glossary

Franz-Xaver -X. Reichl and Gisela Degen

Abuse Improper and excessive use of drugs or stimulants (e.g., alcohol, tobacco) and the use of pharmaceutical products without medical indication or in exceeding doses.

Acceptable Risk This is a risk management term (for a given risk level). Risk levels used for risk evaluations can only be sociopolitically established rather than scientifically substantiated. Numerous criteria have to be taken into account apart from risk perception, e.g., severity of health damage, the possible extent of damage (type of damage and/or number of persons affected), relation to other comparable risks, direct benefit, and actual and possible risk reduction measures. According to a concept adopted in 2007 by the German Committee on Hazardous Substances (AGS), an *acceptable* and a *tolerable* risk level serves to derive exposure-risk relations for carcinogenic chemicals at the workplace. This concept for setting risk-based occupational exposure limits is linked to a set of risk reduction measures.

Accumulation Enrichment of a substance in a medium or environmental compartment. Bioaccumulation is the successive enrichment of a repeatedly administered chemical in an organism when the half-life is very long due to minor metabolization and slow excretion. Accumulation occurs often in specific organs, e.g., cadmium in kidney, lead in bone, and PCDD in fat tissue. Body burden.

Acute Toxicity Adverse effect occurring within a short time of exposure (up to max. 14 days) after a single (high) dose (see also (Sub)chronic Toxicity).

Acute Toxicity Test Test with an observation time up to 14 days after a single dose. The toxic class method (for estimating LD50) requires a clearly reduced number of animals.

F.-X. -X. Reichl
LMU Munich, Munich, Germany
e-mail: reichl@lmu.de

G. Degen
Leibniz Research Centre for Working Environment and Human Factors, Dortmund, Germany
e-mail: degen@ifado.de

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Adaptation Compensatory change in an organism, in response to certain environmental conditions, which occurs without disruption of the biological system and without exceeding the homeostatic capacities of its response.

Added Risk Difference between the incidence of an adverse effect on a treated group of organisms or a group of exposed humans and a control group.

Additive Effect An effect which is the simple sum of the effects of two or more chemicals acting independently (see also Combined Chemical Effects).

ADI (Acceptable Daily Intake) Dose of an agent (amount expressed on a body mass basis) to which an individual in a (sub)population may be exposed daily over its lifetime without an appreciable health risk. The WHO sets ADI values for food additives and tolerable daily intakes (TDI) for contaminants; they are calculated by division of the *NO(A)EL* with a *safety factor* (see also Reference Dose and TDI).

Adverse Effect "Change in the morphology, physiology, growth, development, reproduction or life span of an organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences. Decision on whether or not any effect is adverse requires expert judgement" (according to IPCS/WHO 1994). A biochemical change, functional impairment, or pathologic lesion that affects the performance of the whole organism or reduces an organism's ability to respond to an additional environmental challenge.

Agent Something (chemically, physically, or biologically active principle) capable of producing an effect.

Aggrieved Party Any natural person or legal entity or rather a group of persons whose interests or values can be affected (derogated) by the impact of risks or by risk reduction measures.

Agonist Chemical that can activate a receptor similar to a physiological mediator.

AGS Ausschuss fu€r Gefahrstoffe, the German Committee on Hazardous Substances. The AGS gives advice to the German Federal Ministry of Labour and Social Affairs regarding regulation of workplace chemicals.

AGW Arbeitsplatzgrenzwert: A health-based OEL for a substance in workplace air set by the German Committee on Hazardous Substances (AGS) according to criteria of BekGS 901 and published in the TRGS 900. The AGS evaluates OELs proposed by the German MAK commission, by SCOEL, and by other scientific advisory bodies (DECOS, TLV committee).

ALARA Acronym for "as low as reasonably achievable"; a term from the US Nuclear Regulatory Commission. It means "making every reasonable effort to maintain exposures to ionizing radiation as far below the dose limits as practical, consistent with the purpose for which the licensed activity is undertaken, taking into account the state of technology, the economics of improvements in relation to state of technology, the economics of improvements in relation to benefits to the public health and safety, and other societal and socioeconomic considerations, and in relation to utilization of nuclear energy and licensed materials in the public interest." The ALARA principle is a regulatory tool in the risk management of substances (when a regular risk assessment is not available) (see also Precautionary Principle).

Allergen Any substance that can cause an allergy. Antigens which cause an allergic reaction (hypersensitivity of type I) by stimulating immunoglobulin E (IgE) responses upon contact with skin and/or mucous membranes. Allergens are often compounds, polypeptides, or proteins, the sensitizing potential of which depends on chemical structure and the presence of allergenic determinants (epitopes).

Allergy An (acquired) hypersensitivity disorder of the immune system against environmental (normally harmless) substances. Allergic reactions (to an allergen) involve excessive activation of mast cells and basophils by IgE antibodies. Symptoms occur on the skin, in mucous membranes, and in the respiratory tract (e.g., urticaria, eczema, edema, conjunctivitis, hay fever, asthma) of sensitized individuals.

Ames Assay In vitro assay (developed by Bruce Ames) for the detection of mutagenic effects of chemicals in bacteria (Salmonella test strains). As it reveals mutagenic effects of, e.g., cigarette smoke components and of a high percentage of known mutagenic carcinogenic substances, the Ames assay is usually a starting point in genotoxicity testing.

Aneuploidy Deviation from the number of the normal (euploid) chromosome set by one or several chromosomes.

Annoyance An unpleasant (mental) state that is characterized by such effects as irritation and distraction. Annoyance can result from (various) environmental stimuli (e.g., noise, odor) perceived as unpleasant or pestering by the recipient(s). The property of being easily annoyed is called irritability.

Antagonism The property of a chemical to counteract the effect of another; e.g., in the case of co-exposure to two chemicals when the resulting effect is less than the simple sum of their independent effects (see also Antagonist).

Antagonist Chemical (or drug) which fits into the inactive conformation of a receptor and thereby diminishes or prevents its activation by another chemical, an agonist.

Antigen A substance which elicits a specific immune response (e.g., formation of antibodies) when introduced into an organism.

Antioxidants Substances that inhibit or prevent oxidation processes which result in undesirable changes of biomolecules.

Antitoxins Antibodies (often immunoglobulins of the IgG class) which can neutralize toxins of microbial, plant, or animal origin (e.g., snake venom).

Application Administration of substances to an organism. Common routes of application are: p.o. = per os (via the gastrointestinal tract), s.c. = subcutaneous (injection under the skin), i.m. = intramuscular (in the skeletal muscle), i.p. = intraperitoneal (injection in the abdominal cavity), i.v. = intravenous (injection in veins).

Assessment Endpoint Qualitative/quantitative expression of a specific factor (a response) with which a risk may be associated through an appropriate risk assessment.

Assessment Factor Numerical adjustment used to extrapolate from experimentally determined dose-response relationships to estimate the agent exposure below which an adverse effect is not likely to occur (see also Safety and Uncertainty Factor).

Atopic Persons Individuals with a predisposition for developing an allergy.

Background Burden/Exposure Substance concentrations in biological samples of humans as a result of normal conditions (without known additional exposure).

BAT Value German "Biologischer Arbeitsstoff-Toleranzwert" for biological tolerance value (BLV) for occupational exposures; defined as the maximum permissible quantity of a chemical substance or its metabolites or the maximum permissible deviation from the norm of biological parameters induced by these substances in exposed humans. As with MAK values, BAT values are established on the assumption that persons are exposed at work for at most 8 h daily and 40 h weekly (see also BEI and EKA).

BEI Biological Exposure Indices: used in the USA analogous to BAT values in Germany.

Benchmark Approach/Dose Adjustment of a mathematical model to the data obtained in a study for the dose-response relationship. The benchmark approach is an instrument to determine a *point of departure* for quantitative risk assessments. The dose that leads to an effect with a certain likelihood can be estimated for a defined frequency (for quantal data) or a defined effect measure (for continuous data), i.e., a benchmark response (BMR). This dose is referred to as benchmark dose (BMD). A $_{BMD10}$ indicates the dose at which there is a 10% risk that the effect concerned would likely occur. The reliability of assessing dose-response relationships is quantified by specifying a confidence interval. The value of the lower (generally 90% or 95%) confidence interval is referred to as benchmark dose lower bound (BMDL).

Bioactivation Conversion of xenobiotics (e.g., by enzymes) to biologically reactive, toxic, or carcinogenic metabolites.

Bioassay An assay for determining the potency (or concentration) of a substance that causes a biological change in experimental animals and living systems.

Bioavailability The fraction of a chemical or drug that can be absorbed by the body through the gastrointestinal system, the pulmonary system, or the skin and is systemically available. By definition, when a medication is administered intravenously, its bioavailability is 100%. Upon administration by other routes, its bioavailability generally decreases (due to incomplete absorption and first-pass metabolism) or may vary from person to person.

Biocide A selectively acting toxic substance that is used to destroy harmful organisms (see *Pesticide*).

Biological Limit Values For occupational health purposes, special human biomonitoring (limit) values have been established, such as Biologischer Arbeitsstoff-Toleranzwert (BAT) and Biologische Leitwerte (BLW) by the German MAK commission or Biological Exposure Indices (BEI) by the AGCIH. These values are meant to allow evaluating the risk to an individual's health that results from exposure to a substance at the workplace (by inhalation and/or dermal uptake) and to protect the health of the employee at the workplace.

Biomonitoring In a broader sense, all biological monitoring methods used to investigate the (complex) relationship between external and internal exposure and, thereby, the potential adverse health and environmental effects. In ambient monitoring, living organisms are used as "sensors" in water/sediment quality

surveillance and compliance to detect changes in an effluent or water body and to indicate whether aquatic life may be endangered. In health monitoring, biomonitoring is a general term for the following subcategories: (a) biological monitoring applying biomarkers of exposure such as internal dose or body burden, (b) biochemical effect monitoring with biomarkers of effective dose (e.g., adduct levels and also tissue dose), (c) biological effect monitoring with biomarkers of effect (e.g., micronuclei), and (d) clinical parameters – biomarkers of disease. Most common in human biomonitoring are studies with biomarkers of exposure and biochemical effects aimed to establish distribution of exposure among the general population (including trends and changes in exposure), identify vulnerable groups and populations with higher exposures, identify new chemical exposures, and identify environmental risks at specific contaminated sites or at workplaces.

Biotransformation Enzymatic conversion of xenobiotics in an organism; metabolization (biotransformation) usually results in products that are less toxic, more water soluble, and readily excreted from the organism. But with some chemicals, biotransformation results in bioactivation and thus an increased toxicity.

Body Burden The total amount of a substance in the body. Some substances build up in the body because they are stored (e.g., in fat or bone) or because they leave the organism very slowly. In such cases, the blood concentration does not reflect the amount stored in the body. Body burden must be measured with independent methods.

Brownfields Sites or soil polluted with hazardous substances (e.g., abandoned or existing waste deposits and/or production sites).

Cancer Disease which results from the development of a malignant tumor and its spreading into surrounding tissues.

Carcinogen An agent capable of inducing cancer. A substance or mixture (e.g., coal tar) which causes tumors (cancer) in animals or humans.

Carcinogenesis The development of cancer, a disease of heritable, somatic mutations affecting cell growth and differentiation, characterized by an abnormal, uncontrolled growth of cells. In chemical carcinogenesis, several steps are defined: initiation, promotion, and progression. Any chemical which can cause cancer is said to be carcinogenic.

Carcinogenicity Test A form of chronic toxicity testing directed to detect carcinogenic effects of chemicals: The test substance is applied to rodents for 18–24 months, usually five times a week, at several dose levels. The appearance of tumors is assessed upon necropsy and compared to the frequency in a nonexposed control group.

Cell Culture The maintenance and propagation of previously isolated cells in a suitable nutrient (culture) medium. Cell cultures are used for various in vitro toxicity tests. Other than primary cells, cell lines can be kept in culture for long periods and passaged numerous times.

Chromosomal Aberration An abnormality in chromosome number or structure.

Chromosome The heredity-bearing gene carrier in the cell nucleus, composed of DNA and protein.

Chronic Toxicity Refers to long-term adverse effects in an organism after dosing of a toxicant over an extended time period. Long-term effects relate to changes in, e.g., growth, reproduction, or the ability to survive. Examples in humans are cardiovascular diseases and cancer from smoking and liver disease from alcohol abuse.

Chronic Toxicity Test Experimental studies with repeated application of a substance over an extended period of time (at least 3 months), usually at several dose levels, to gain information on, e.g., organ toxicity, tumor formation, and dose-effect relationships.

Clastogens Agents which cause chromosomes to break. This may be a result of direct damage to the DNA or by indirect mechanisms, e.g., inhibition of topoisomerases.

Clearance The process of losing a substance from the body. Total clearance (Cltot) is a measure for the ability of an organism to eliminate a given substance by both renal and extrarenal clearance. Renal clearance (ClR) is a function of glomerular filtration, secretion from the peritubular secretion of the nephron, and reabsorption from the nephron back to these blood vessels. Another major route for elimination of foreign compounds is their uptake by liver cells and secretion into bile.

CLP Acronym for Regulation on **C**lassification, **L**abelling, and **P**ackaging of chemical substances and mixtures (see also ECHA and REACH).

Combined Chemical Effects Chemicals that act by the same mode of action and/or at the same target cell or tissue often act in a (potency-corrected) "dose-additive" manner. Where chemicals act independently, by discrete modes of action or at different target cells or tissues, the effects may be additive ("effects additive" or "response additive"). Alternatively, chemicals may interact to produce an effect, such that their combined effect "departs from dose additivity." Such departures comprise "synergy," where the effect is greater than that predicted on the basis of additivity, and "antagonism," where the effect is less than that predicted on the basis of additivity. Related terms are "mixture toxicity," additive effect, antagonism, and synergism.

Compartment In pharmaco- and toxicokinetics, a compartment is a defined volume of body fluids. Major body compartments are blood plasma, interstitial fluid, fat tissue, and intracellular and transcellular fluid. With the exception of blood, where the volume is rather well defined, other "compartments" are of less distinct size, because the volume for distribution of a given substance can comprise various body fluids and tissues. In pharmaco-/toxicokinetics, "compartments" are separated entities which have a defined volume and defined rates of influx and efflux. These interact with each other in a dynamic way. There are one-, two-, or multi-compartment mathematical models. The models are a practical approach to a much more complex reality.

Concentration-Effect Relationship Relationship between the exposure, expressed in concentration, of a given organism, system, or (sub)population to an agent in a specific pattern during a given time and the magnitude of a continuously graded effect to that organism, system, or (sub)population.

Concern Level Concentration of an environmental chemical expected/suspected to cause harm to a population in field experiments.

Congeners Substances whose structure, function, or origin is similar to others and may match the same structure-activity relationship (SAR). Examples are polyhalogenated dibenzodioxins and -furans (dioxins) which can have diverse toxicological properties.

Consumer Protection All areas of legislation and policy which serve to protect citizens (private persons) who are buying or consuming goods or demanding services. Protecting the health of consumers involves mainly issues of food safety, product safety (e.g., personal care products, detergents and household cleaners, textiles, toys), and other consumer goods.

Contamination In general, the presence of a minor or unwanted constituent (contaminant) in a material (physical or body tissue), the environment, at a workplace, etc. In food and medicinal chemistry, the term contamination usually refers to the presence of toxic substances or pathogens.

Course of Action Variants of possible actions in risk management to reduce risks, including the option for nonaction (for minimal risks). In the case of action, there may be also options for different risk reduction measures.

Cross-reactivity Immunological reaction of specific antibodies or specifically sensitized T-lymphocytes with compounds having similar or identical determinants as the so-called homologous antigen.

Cytochrome P-450 A family of heme containing enzymes that transfer oxygen to chemicals (old term mixed-function oxidases) involved in phase I reactions of xenobiotics. They are located on microsomes and have a light absorption peak near 450 nm.

Cytotoxicity Ability of an agent to cause disturbance to cellular structure or function, often leading to cell death.

DDT Dichloro-diphenyl-trichloroethane (Chlorphenotan). Contact insecticide, now widely banned because of its high persistence in the environment and accumulation in the food chain.

Decontamination Removal of hazardous substances, e.g., from materials, from soil, or from dead and living tissues.

Default Statistically supported standard value or assumption that is to be used in the absence of substance-specific or species-specific data. A default is a means to describe systems despite incomplete knowledge of their characteristics.

Deposition Sedimentation of solid, liquid, or volatile particles in the organism.

Desoxyribonucleic Acid That constituent of cells which stores the hereditary information of an organism in the form of a sequence of nitrogenous bases. Much of this information relates to the synthesis of proteins. Damage of DNA can result in a mutation.

Detergent A cleaning or wetting agent which possesses polar and nonpolar functional groups or surfaces allowing interaction with nonpolar molecules making them miscible with a polar solvent.

Detoxification (a) A process which renders a toxic molecule less toxic by biotransformation, removal, or the masking of active functional groups, and (b) the

treatment of patients suffering from poisoning in order to reduce the probability or severity of harmful effects.

Dioxin(s) Systematic term for a twofold unsaturated six-membered ring system with two oxygens in the ring. Dioxin is used colloquially for the group of polychlorinated dibenzodioxins (PCDD) and sometimes also the polychlorinated dibenzofurans (PCDF); in the first group, there are 75, and in the latter, 135 isomers (congeners). The most famous dioxin, the "Seveso-poison," i.e., 2,3,7,8-tetrachlordibenzo[1,4,]dioxin (2,3,7,8-TCDD) is far more toxic than all other congeners.

Disinfectants Substances/preparations used to reduce or eliminate (pathogenic) microorganisms on skin and other surfaces. Examples are ethanol, phenol, soaps, and tensides which act against bacteria.

Distribution Dispersal of a xenobiotic and its derivatives throughout an organism or environmental matrix, including tissue binding and localization. In toxicokinetics, this includes the passage of a substance from one compartment (e.g., blood, extracellular fluid) to another (e.g., fat tissue), moving towards an equilibrium.

Dose Total amount of an agent administered to, taken up, or absorbed by an organism, system, or (sub)population. Administered doses are often given in mg/kg of body weight.

Dose-Effect Relationship The (functional) relationship between the dose and the magnitude of a continuously graded effect in an organism, system, or (sub) population (see also Dose-Response Relationship).

Dose-Response Relationship Relationship between the total amount of an agent (the dose) and responses in an organism, system, or (sub)population in reaction to that agent (see also Dose-Effect Relationship).

Dust Fine, dry powder consisting of inorganic particles (e.g., ash, clay, rock chip, sand) and/or organic material (e.g., fungal spores, microorganisms, mites, feather or plant fragments, sooty particles), matter lying on the ground or on surfaces or carried in the air. Dusts are generated by work processes such as cutting, crushing, detonation, grinding, and handling of organic and inorganic matter such as coal, grain, metal, ore, rock, and wood, but may also occur naturally (e.g., pollens, volcanic ashes, sandstorms). The term "airborne dust" often refers to airborne particulate matter ranging in diameter from 1 to 100 mm, which differs in deposition in the respiratory tract. Very small particles (fine and ultrafine, less than 5 mm) are of concern as they deposit in the tracheobronchal and alveolar regions. Fibrous dusts, such as asbestos and other such materials, have been shown to present special health problems primarily related to the shape of the particles (see also Fibers).

EC50 Effective concentration which affects 50% of a test population after a specified exposure time.

ECHA European Chemical Agency (in Helsinki, Finland) with the task of implementing the EU's chemical legislation. The mission of ECHA is to manage all REACH and CLP tasks by carrying out or coordinating the necessary activities, ensure a consistent implementation at the community level, and provide

member states and the European institutions with the best possible scientific advice on questions related to the safety and the socioeconomic aspects of the use of chemicals.

Ecotoxicology The study of effects of toxic chemicals on biological organisms, mainly at the population, community, and ecosystem levels. A multidisciplinary field which integrates toxicology and ecology, with the aim to predict the effects of pollution and to gather information as to the best course of action to restore already affected ecosystems. Ecotoxicology differs from environmental toxicology in that it integrates the effects of stressors across all levels of the biological organization, whereas environmental toxicology focuses upon effects at the level of individual species and the occurrence and fate of anthropogenic chemicals in the environment.

ECVAM European Centre for the Validation of Alternative Methods.

ED50 Dose that affects a designated criterion in 50% of the population observed. Also known as median effect concentration/dose.

Effect A change in the state or dynamics of an organism, system, or (sub)population caused by exposure to an agent.

EIA Environmental Impact Assessment.

EINECS European Inventory of Existing Commercial Substances.

EKA Value Exposure equivalents for carcinogenic substances at the workplace (see also BAT Value).

Elimination The combined process of metabolism and excretion which results in the removal of a substance from an organism.

ELINCS European List of Notified Chemical Substances.

Embryotoxicity Damage to the embryo (the undeveloped animal or individual), e.g., by chemicals, which results in early death, delays in development, impaired organ function, or malformations (see also Teratogenicity).

Emission Release of a substance (or radiation) from a source, including discharges into the wider environment.

Endogenous Arising within or derived from the organism.

Environmental Health A branch of public health related to all aspects of the natural and built environment that may affect human health. The term "environmental hygiene" is used synonymous with environmental health.

Environmental Impact Assessment EIA: A procedural step – introduced by an amendment to Federal Act on EIA in 2001 in Germany – for a screening process ("case-by-case examination") in order to determine whether for a given project (e.g., new streets, train tracks) an EIA is required in the authorization procedure. With regard to chemicals, toxicity testing in nonmammalian species is part of the required hazard assessment under ecological aspects.

Environmental Medicine A field of medicine which studies the interaction between environment and human health and the role of the environmental factors in causing or mediating disease in patients (i.e., in a clinical setting) and, thereby, differs from environmental health.

Environmental Protection A wide range of societal and individual "measures" aimed to prevent or remediate interferences with ecosystems, e.g., in raising

consciousness by information campaigns, labeling of "eco-friendly" products, or setting of standards for hazardous chemicals and pollutants or use restrictions and bans of particularly hazardous agents (such as DDT, vPvB).

Environmental Toxicology A field of toxicology which focuses upon the occurrence and fate of anthropogenic chemicals in the environment and the effects of pollutants at the level of individual species (see also Ecotoxicology).

EPA Environmental Protection Agency (in the USA, also in Denmark and elsewhere; in Germany, UBA).

Epidemiology The study of the incidence, distribution, and causes of disease or the statistical study of categories of persons and the patterns of diseases from which they suffer in order to determine the events or circumstances causing the diseases.

Epoxide Highly reactive metabolites with the ability to bind to cell components. Epoxides are held responsible for the toxic and carcinogenic effects of, e.g., polycyclic aromatic hydrocarbons and certain other organic compounds.

Excretion Removal of a substance or its metabolites from an organism by the discharge of biological material, including urine, feces, expired air, mucus, milk, eggs, and perspiration.

Existing Chemicals Chemicals which have been available in an EC member state between 1971 and 1981 – prior to the introduction of legal obligations for testing them for hazardous properties – and have been listed in EINECS (see also REACH).

Exogenous Resulting from events or derived from materials external to an organism.

Expert Judgment The opinion of an authoritative person on a particular subject.

Exposure The concentration or amount of a particular agent that reaches a target organism, system, or (sub)population at a specific frequency for a defined duration.

Exposure Assessment Evaluation of the exposure of an organism, system, or (sub) population to an agent (and its derivatives). Exposure assessment is a key step in the process of risk assessment.

Exposure Routes External routes by which a chemical exposure of the organism can occur (e.g., by inhalation, dermal contact, or oral intake) from air, food, water, or soil.

Extrapolation An estimation of a numerical value of an empirical (measured) function at a point outside the range of data used to calibrate the function or the use of data derived from observations to estimate values for unobserved entities or conditions....

Fecundity (1) Potential to produce offspring frequently and in large numbers, and (2) in demography, the physical ability to reproduce. A lack of fecundity is called sterility.

Fertility In humans and mammals, the natural capability to produce offspring. Fertility in a stricter sense differs from fecundity which is defined as the potential for reproduction and influenced by gamete production (sperm, eggs), fertilization, and carrying a pregnancy to term.

Fetotoxicity Damage to mammals in the womb, after completion of organogenesis. In humans, this stage is reached after about 3 months of pregnancy. Prior to this, the developing mammal is in the embryo stage (see also Embryotoxicity).

Fibers In relation to health, particles with a diameter < 3 mm, length > 5 mm, and aspect ratio (length to width) greater than or equal to 3 to 1 are classified as "fibers" (WHO 1997). Examples of fibers include asbestos (comprising two groups of minerals: the serpentines, e.g., chrysotile, and the amphiboles, e.g., crocidolite – "blue asbestos"). Other examples include synthetic fibrous materials, such as rock wool (or stone wool) and glass wool, as well as ceramic, aramid, nylon, and carbon and silicon carbide fibers.

First-Pass Effect The metabolism that an ingested compound undergoes in its passage through the gut and liver before reaching the systemic circulation.

Fly Ash (Or flue-ash): Residues generated during combustion of coal which comprise fine particles that rise with the flue gases. Depending on the specific type of coal burnt, fly ash can contain highly toxic materials (arsenic, heavy metals, along with dioxins and PAH); these are concentrated in filter devices (which have to be treated as hazardous waste).

Food Chain A sequence of links in a food web between different trophic species, starting, for instance, with basal species such as producers of fine organic matter (plants) and continuing with consumer organisms (herbivores and carnivores). Persistent lipophilic substances (e.g., pesticides) can accumulate in the course of the food chain (see Bioaccumulation).

Genotoxicity Ability to cause damage to the genetic material or an adverse effect in the genome, e.g., a gene mutation, chromosomal aberration, or aneuploidy. Genotoxicity is a broader term and refers also to processes which alter the genetic material, yet are not necessarily associated with mutagenicity. Thus, tests for genotoxicity include tests which provide an indication of induced damage to DNA (but no direct evidence of mutation), e.g., increases in unscheduled DNA synthesis or sister chromatid exchange, DNA strand breaks, DNA adduct formation, as well as tests for mutagenicity.

GHS The Global Harmonized System for classification and labeling of chemicals (CLP) of the UN, implemented under Regulation (EC) No. 1272/2008.

GLP Good Laboratory Practice, a quality assurance system.

Guidance Value Value, such as concentration in air or water, which is derived after allocation of the reference dose among the different possible media (routes) of exposure. The aim of the guidance is to provide quantitative information from risk assessment to risk managers to enable them to make decisions (see also Reference Dose).

Half-life The time interval (commonly denoted as $_{t1/2)}$ that corresponds to a concentration decrease by a factor of 2. After five half-lives, the blood level will be 3% of the initial concentration, a decrease due to metabolism or excretion. Compounds with a long half-life can accumulate upon repeated intake. Environmental half-life data generally reflect the rate of disappearance from a medium without identifying the mechanism of chemical loss.

Half-life, Biological Time interval after administration of a substance to an organism in which half of the originally present dose is eliminated, i.e., metabolized or excreted.

Harm Physical injury or mental damage; actual or potential ill effects or danger. For example, "smoking when pregnant can harm your baby."

Hazard (1) A potential source of harm. (2) The inherent property of an agent or a situation having the potential to cause adverse effects in organisms or individuals.

Hazard Assessment A process designed to determine the possible adverse effects of an agent (or situation) to which an organism could be exposed. The process includes hazard identification and hazard characterization. The process focuses on hazard in contrast to *risk assessment* where exposure assessment is a distinct additional step.

Hazard Characterization The qualitative and, wherever possible, quantitative description of the inherent properties of an agent (or situation) having the potential to cause adverse effects. It is a stage in hazard assessment and the second step in risk assessment (see also Concentration/Dose-Effect Relationship).

Hazard Identification The identification of the type and nature of adverse effects that an agent can elicit in an organism. Hazard identification is the first stage in the process of hazard assessment and the first in the process of risk assessment.

Hazardous Situation Circumstances of danger for a given object (see also Risk).

HBM Value Human biomonitoring values I and II

HCH Acronym for six chlorine substituted cyclohexanes; the most common form is gamma-HCH (lindane).

HCp (HC5) Value Hazardous concentration for p % (5%) of the species, derived by means of statistical extrapolation for acceptable levels in ecotoxicology.

Health As defined by the WHO, health is a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity.

Health Damage/Impairment All temporary or permanent undesirable changes triggered by, e.g., chemicals, radiation, accidents, or lifestyle factors.

Health Protection All measures taken to protect the health and well-being of the population against harmful factors, such as hazardous chemicals, infectious agents, and radiation. Preventive health protection aims to keep the possible impact low by means of exposure reduction and education measures.

Health Risk The probability (likelihood) that damage to health will occur in a population with exposure to a harmful agent or factor. This depends upon the intensity and duration of exposure to a hazardous compound or factor and its activity/affectivity.

HPVC High Production Volume Chemical.

Human Equivalent Dose The human concentration (for inhalation exposure) or dose (for other routes of exposure) of an agent that is believed to induce the same magnitude of toxic effect as the experimental animal species concentration or dose. This adjustment may incorporate toxicokinetic information on the particular agent, if available, or use a default procedure, such as assuming that daily oral doses experienced for a lifetime are proportional to body weight raised to the power of 0.75.

Human Biomonitoring Values I and II (HBM I and II) The Human Biomonitoring (HBM) Commission of the German Federal Environmental Agency (UBA) defines two different types of HBM values: HBM I and HBM II. HBM I describes the concentration in the body matrix of a substance below which, according to the Commission's current assessment, no adverse health effect should be expected. The HBM II value represents the concentration above which there is an increased risk for adverse health effects; it is thus regarded an intervention or "action" level. HBM I and II values are set on the basis of health risk assessments and are thereby clearly distinct from reference values (RV95). RV values (derived according to a defined statistical method from a series of analytical results) are statistical descriptions of the ranges of concentrations typically seen in a specified reference population, but which have no direct relationship to health effects or risk assessment.

i.v. Intravenous administration (injection).

ICH The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. A source for toxicity test guidelines in drug development.

Immunotoxicity Adverse effects of chemicals (or other agents) on components/function of the immune system.

In Vitro/In Vitro Test In glass; refers to studies and tests in the laboratory usually involving isolated organs, tissues, cells, or biochemical systems.

In Vivo/In Vivo Test Within the living organisms; refers to studies and tests of chemicals in laboratory animals.

Incidence The number of newly diagnosed cases of a certain disease within a given period of time; epidemiological measure to characterize disease trends in a certain population.

Incidence Rate The ratio of new cases within a population to the total population at risk given a specified period of time.

Incorporation The entry/uptake of a (hazardous) substance in an organism or a compartment (e.g., lung, gastrointestinal tract).

Interest Group Parts of the population organized as a group that holds and presents a common view.

Interspecies Dose Conversion The process of extrapolating from animal doses to human equivalent doses.

Intervention Value A value discussed in the context of chemical residues and contaminants of food which is lower than the respective (maximal) limit values. When the intervention value is exceeded, appropriate measures should be taken to reduce emissions of this substance in the environment and thereby prevent contamination of food commodities.

Intolerance (Med.) Varied (nonallergic) responses to drugs (drug sensitivity) or food ingredients (e.g., lactose)....

Intoxication (Poisoning): Impact of usually chemically defined, toxic agents. These substances may also be of mineral, plant, animal, or viral origin. They can "enter" the body via the gastrointestinal tract, the respiratory system, the intact skin, but also via wounds or injection. The severity of an illness depends upon the toxicity,

the amount (dose), the duration of exposure, and the susceptibility of the afflicted individual. Poisoned persons often show typical symptoms.

Invasion Passage of an incorporated substance into the circulation (e.g., blood or lymph); resorption and distribution in the body.

IPCS International Programme on Chemical Safety of the WHO.

IRIS Acronym for Integrated Risk Information System. More information (list of substances and documents/reviews) is available on the EPA page: http://www.epa.gov/iris/intro.htm

Irritants Substances which cause local reactions (e.g., erythema) on skin or mucous membranes upon prolonged contact due to irritant properties. Agents with corrosive properties (e.g., strong acids or bases) cause more severe damage.

JECFA Joint FAO/WHO Expert Committee for Food Additives (and Veterinary Drugs and Contaminants). An international committee that sets ADI and TDI values.

Latency Period The time between first exposure to an agent and manifestation or detection of a health effect of interest.

LC50 (Lethal Concentration) Concentration of an agent in the surrounding atmosphere, respectively for aquatic organisms in water, which results in the death of 50% of the exposed individuals.

LD50 (Lethal Dose) The median lethal dose that is estimated to cause death of 50% of the test organisms.

Limit Values Limit values (maximal permissible concentrations) for hazardous chemicals are set to protect humans and the environment against harmful effects. Examples of toxicologically founded limit values are maximal residual levels for pesticide contaminants in food or MAK values and other OELs for industrial chemicals at the workplace. Limit values are quantitative standards where noncompliance triggers legal consequences while a "guidance" value has to be observed only when this is possible.

Lindane 1,2,3,4,5,6-Hexachlocyclohexane (gamma-HCH), a pesticide.

Linear Dose Response A pattern of frequency or severity of biological response that varies directly with the amount of dose of an agent.

LO(A)EL (Lowest Observed (Adverse) Effect Level) LOEL: In a study, the lowest dose or exposure level at which a statistically or biologically significant effect is observed in the exposed population compared with an appropriate unexposed control group. LOAEL: The lowest exposure level at which there are biologically significant increases in frequency or severity of adverse effects between the exposed population and its appropriate control group.

LOEC The lowest concentration at which a statistically or biologically significant effect is observed in the exposed population compared with an appropriate unexposed control group.

MAC and MAK Value Regulatory value (MAC) defining the concentration which if inhaled daily (for workers: 8 h/day over a working week of 40 h; for the general population: 24 h/day) does not appear capable of causing appreciable harm in the light of the present knowledge. In Germany, MAK values (*Maximale*

Arbeitsplatz-Konzentration) for volatile chemicals and dusts are proposed by the Senate Commission of the German Research Foundation Council (DFG) on the basis of toxicological data and workplace-related observations. The MAK Commission also draws up proposals for BAT values (biological tolerance values) and develops procedures to analyze chemical substances in the air and in biological materials. The list of MAK and BAT values is published annually and presented to the German Federal Ministry of Labour and Social Affairs. The Ministry's Committee on Hazardous Substances (AGS) subsequently reviews the proposals and makes recommendations for their inclusion in the Hazardous Substances Ordinance.

Malignant Tumor An abnormal growth of tissue which can invade adjacent or distant tissue.

Margin of Exposure (MOE) Ratio of the no observed adverse effect levels (the NOAEL or other point of departure) for the critical effect to the theoretical, predicted, or estimated dose or concentration. The MOE is a tool used by risk assessors to consider possible safety concerns arising from the presence in food and feed of substances which are both genotoxic (i.e., which may damage DNA, the genetic material of cells) and carcinogenic (see also Margin of Safety (for noncarcinogenic substances)).

Margin of Safety (MOS) For some experts, the MOS has the same meaning as the MOE (margin of exposure), while for others, the MOS means the margin between the reference dose and the actual exposure dose or concentration.

Mechanism of Action The interpretation of pharmacological or toxicological effects on the basis of biochemical and molecular data; a sufficient understanding of the molecular basis for an effect so that causation can be established. Compare Mode of Action.

Medicine The applied science or practice of the diagnosis, treatment, and prevention of disease.

Metabolic Activation See Bioactivation.

Metabolism In toxicology, the term refers to the conversion of xenobiotics by endogenous enzymes (see Biotransformation).

Metabolite The product from a biotransformation process of xenobiotics.

Minimal Risk Level An MRL is an estimate of the daily human exposure to a hazardous substance that is likely to be without appreciable risk of adverse noncancer health effects over a specified duration of exposure. The substance-specific estimates, which are intended to serve as screening levels, are used by the US Agency for Toxic Substances and Disease Registry (ATSDR) health assessors and other responders to identify contaminants and potential health effects that may be of concern at hazardous waste sites.

Mode of Action A postulated MoA for a chemical is a plausible sequence of key events leading to an observed effect, supported by robust experimental observations and mechanistic data. Both MoA and mechanistic data can be important elements in chemical risk characterization, e.g., with regard to the question whether an adverse effect is thresholded or not.

MOE See Margin of Exposure.

Monitoring Repeated measurements, observations, and evaluation of specified properties of the environment, in order to define the current state and establish trends over time. Surveys and surveillance are used to achieve this objective and/or study the situation after taking measures to reduce risk, e.g., from contaminants.

Morbidity The state of disease or illness within a population; the morbidity rate is given as incidence and prevalence. Compare also Mortality.

Mortality (Rate) The state of death; mortality rate is a measure of the number of deaths (in general or due to a specific cause) in a population, scaled to the size of that population, per unit of time.

MRL See Minimal Risk Level.

Mutagenicity The ability of a substance (or agent) to induce a *mutation*.

Mutagenicity Testing Studies on mutagenic properties of chemicals: Several in vitro assays (tests in bacteria, such as Ames assay and mammalian cells) or in vivo assays are used for the identification of agents that can induce or increase the frequency of mutation in an organism (see also Genotoxicity).

Mutation A change in the amount or structure of the genetic material (DNA) of cells or organisms. Changes may involve a single gene or gene segment, a block of genes, or chromosomes. Mutagenicity refers to the induction of permanent transmissible changes either in somatic cells or in germ cells (see also *Genotoxicity*).

N(O)EC No (observed) effect concentration. The highest concentration of a material or substance in a toxicity test that has no statistically significant adverse effects on the exposed population of test organisms compared with the control group.

NAS National Academy of Science in the USA.

Necrosis Cell death or death of areas of tissue, usually surrounded by healthy tissue. Necrosis (caused by trauma or toxic chemicals) results in an unregulated digestion of cell components. It differs from apoptosis, a programmed and targeted cause of cell death in multicellular organisms.

Neurotoxicity Toxicity on any aspect of the central or peripheral nervous system. Adverse effects may be observed as functional changes (behavioral or neurological abnormalities) or as neurochemical, biochemical, physiological, or morphological changes.

NOAEL No observed adverse effect level. The highest exposure level at which there are no biologically significant increases in the frequency or severity of adverse effects between the exposed population and its appropriate control group; some effects may be produced at this level, but they are not considered adverse or precursors of adverse effects.

Nocebo In medicine, a nocebo reaction is the harmful, unpleasant, or undesirable effect seen in a subject who received an inert tablet (e.g., sugar pill) and may be also observed in persons with exposure to chemicals at irrelevant levels. Nocebo responses are due to the subject's pessimistic belief and expectation that the inert drug (or the chemical) will produce negative consequences. The opposite of *placebo*.

NOEL No observed (adverse) effect level. An exposure level at which there are no statistically or biologically significant increases in the frequency or severity of any effect between the exposed population and its appropriate control group.

Notified Substance According to REACH, a substance for which a notification has been submitted and which could be placed on the market in accordance with Directive 76/548/EEC. In the EC, "new chemicals" were those produced since 1981 and not listed on the EINECS.

Objective (Protection Goal) The goal to be reached with respect to a desired level of protection. The framework for risk regulation often sets the objective, i.e., a level of protection adequate for averting a danger (i.e., hazard control) or for prevention.

Occupational Disease An official list of diseases which have been recognized to be caused by specific influences at the workplace. The list is used – along with several criteria for causation (i.e., occupational history, conditions at the workplace, exposure measurements) – in legal procedures to establish (or refute) cases claiming compensation.

Occupational Medicine Applied science dealing with occupational diseases, workplace accidents, questions of occupational hygiene, and toxicology.

Occupational Safety All measures to protect workers against accidents and diseases in conjunction with the workplace. Important elements of occupational safety are on-the-job safety requirements and company medical doctors. OEL and MAK values limit exposure to hazardous chemicals.

Odds Ratio (OR) A relative measure of the difference in exposure between the diseased (cases) and not diseased (controls) individuals in a case-control study. The OR is interpreted similarly to the relative risk.

OEL Occupational Exposure Limit; expressed as concentration of a substance in the air at the workplace.

Oral Slope Factor An upper bound, approximating a 95% confidence limit, on the increased cancer risk from a lifetime oral exposure to an agent. This estimate, usually expressed in units of proportion (of a population) affected per mg/kg-day, is generally reserved for use in the low-dose region of the dose-response relationship, i.e., for exposures corresponding to risks less than 1 in 100.

Overadditive Effects See Synergism.

PAH Polycyclic aromatic hydrocarbons.

PBPK Physiologically Based Pharmacokinetic Model. A model that estimates the dose to a target tissue or organ by taking into account the rate of absorption into the body, distribution among target organs and tissues, metabolism, and excretion.

PCB Polychlorinated Biphenyls. An important group of environmental chemicals, some of which tend to persist in the environment and to accumulate in the human body.

PCDD Acronym for poly-chlorinated dibenzo-1,4-dioxins; a class of 75 chemicals with several highly toxic compounds. The best known example of these ubiquitously found "environmental chemicals" is 2,3,7,8-tetrachloro-dibenzo-1,4-dioxin, colloquially termed dioxin.

PCDF Polychlorinated dibenzofurans (see also PCDD and Dioxin).

PCP Pentachlorophenol, previously widely used for preservation of wood, textiles, and leather and as disinfectant.

PEL Permissible Exposure Limit. In the USA, a legal limit for exposure of an employee to a chemical substance or a physical agent. PEL values, established by OSHA, are usually expressed in ppm or mg/m^3 (see also MAC/MAK values).

per os (p.o.) Administration via the mouth, e.g., by gavage (bolus) or with food.

Pesticide(s) Chemicals used in agriculture and other areas to control the severity and incidence of pests and diseases. Pesticides (or "biocides") are used to control bacteria, fungi, algae, higher plants, nematodes, molluscs, mites and ticks, insects, rodents, and other organisms. The generic term is also used to cover bactericides, fungicides, algicides, herbicides, nematocides, molluscicides, acaricides, insecticides, and rodenticides.

Pharmacodynamics See Toxicodynamics.

Phase-I (Reactions) First step in biotransformation. Modification of a molecule (drug or other chemical) by oxidation, reduction, hydrolysis, hydration, dechlorination, or other reactions which are catalyzed by enzymes, mainly xenobiotic metabolizing enzymes of the endoplasmic reticulum (microsomal) or cytosolic enzymes (see also Cytochrome P450).

Phase-II (Reactions) Step in the biotransformation of a substance or its phase-I reaction product by enzyme families which catalyze either acetylation, glucuronidation, sulfation, or conjugation with glutathione. This results in the formation of more water-soluble metabolites which can be excreted in urine or bile.

Placebo Latin: "I shall please." A simulated treatment or an inert drug given in medical research as control treatment. Interestingly, patients receiving a placebo may feel or show an (actual) improvement in their condition, a phenomenon known as the "placebo effect." The opposite phenomenon is known as nocebo effect.

Point of Departure (POD) The dose-response point that marks the beginning of a low-dose extrapolation. This point can be the lower bound on dose for an estimated incidence or a change in response level from a dose-response model (BMD), or a NOAEL or LOAEL for an observed incidence or a change in response level (see also Benchmark Approach/Dose).

Point Source Emission source(s), either single or multiple, which can be quantified by means of location and the amount of substance emitted per source and emission unit (e.g., amount per time).

Poison Compound of which a certain dose can elicit toxic effects and, possibly, death. A poison may be a mixture of various toxic substances: Natural poisons (e.g., bee or snake venom) are often secretion products and consist of numerous components, i.e., toxins and/or (toxic) enzymes as well as poisonous plant extracts.

Pollution Release to or presence in the environment of a chemical, physical, or biological agent (a pollutant) that has the potential to damage the health of humans or other organisms.

Polymorphism The existence of interindividual differences in DNA sequences coding for one specific gene. The effects of such differences may vary, from fully intact protein to inactive protein or lack of synthesis of this protein. In the context of pharmacology and toxicology, genetic polymorphisms of xenobiotic metabolizing enzymes are of special interest, as certain (iso)enzymes which are expressed at higher or lower levels or missing in individuals may predispose them to adverse effects (see also Susceptibility and Biotransformation).

POP(s) Persistent organic pollutant(s). PCDDs, PCDFs, and PCBs are persistent organic pollutants (POPs) under the Stockholm Convention on Persistent Organic Pollutants (http://www.pops.int/) and are omnipresent in the global environment.

ppb A unit of measure expressed as parts per billion. Equivalent to 1×10^{-9}, e.g., mg per kg.

ppm A unit of measure expressed as parts per million. Equivalent to 1×10^{-6}, e.g., mg per kg.

Prevalence The proportion of disease cases that exist within a population at a specific point in time, relative to the number of individuals within that population at the same point in time.

Prioritization Setting priorities in the sequential risk assessment of (numerous) agents under consideration according to their perceived importance.

Probability A quantitative statement about the likelihood of a specific outcome. Probability values can range from 0 to 1.

Proliferation Multiplication, i.e., an increase by frequent and repeated reproduction or growth by cell division.

Promotion Phase of proliferation of carcinogen-initiated cells in the context of carcinogenesis.

Promotor An agent that is not carcinogenic in itself, but when administered after an initiator of carcinogenesis stimulates the clonal expansion of the initiated cell to produce a neoplasm.

PTWI (Provisional Tolerable Weekly Intake) The weekly dose of a contaminant which an individual may ingest over its lifetime without an appreciable health risk, according to current knowledge (thus provisional). The WHO sets PTWI values for food contaminants (see also ADI and TDI).

QSAR Quantitative structure-activity relationship. A quantitative relationship between a biological activity (e.g., toxicity) and one or more molecular descriptors that are used to predict the activity (see also Structure-Activity Relationship).

REACH Registration, Evaluation, Authorisation and Restriction of Chemicals – the new chemical legislation of the European Union (Regulation No. 907/2006) which came into force in 2007. It replaces about 40 directives and regulations and erases the former regulatory distinction between newly notified substances and existing chemical substances. REACH does not touch special regulations for drugs, biocides, radioactive compounds, and food and feed additives.

Reference Dose Acronym: RfD. "An estimate (with uncertainty perhaps spanning an order of magnitude) of a daily oral exposure to the human population (including sensitive subpopulations) that is likely to be without an appreciable risk of deleterious effects during a lifetime. It can be derived from a NOAEL, LOAEL,

or benchmark dose, with uncertainty factors generally applied to reflect limitations of the data used" (WHO/IPCS 2004) (see also Reference Values).

Reference Values (RfD or RfC and RV95) An estimate of an exposure for a given duration to the human population (including susceptible subgroups) that is likely to be without an appreciable risk of adverse health effects over a lifetime. It is derived from a BMDL, a NOAEL, a LOAEL, or another suitable point of departure, with uncertainty/variability factors applied to reflect limitations of the data used. Reference value, a generic term as used by EPA (2002), is not specific to a given route of exposure. EPA develops numerical toxicity values for the reference dose (RfD) and reference concentration (RfC), usually for non-cancer health assessments. The term "reference value" is also used in human biomonitoring: Here, $_{RV95}$ are statistical descriptions of the range of concentrations typically seen in a specified reference population, but which have no direct relationship to health effects or risk assessment. But, $_{RV95}$ are an important tool for prevention to assess whether populations (or individuals) are more exposed when compared to the environmental background exposure. Compare also Human Biomonitoring Values I and II (HBM I and II).

Relative Risk The relative measure of the difference in risk between the exposed and unexposed populations in a cohort study. The relative risk is defined as the rate of disease among the exposed divided by the rate of disease among the unexposed. A relative risk of 2 means that the exposed group has twice the disease risk as the unexposed group.

Reproductive Toxicology The study of the adverse effects of chemicals (and medicinal drugs) on the embryo, fetus, neonate, and prepubertal animal and the adult reproductive and neuroendocrine systems. Reproductive toxins comprise both agents which impair the fertility of adult organisms and/or those which can adversely affect the developing organisms (see also Embryotoxicity and Teratogenicity).

RfD See Reference Dose.

Risk The probability of an adverse effect in an organism, system, or (sub)population caused under specific circumstances, e.g., by exposure to an agent and/or a situation.

Risk (Human Health) The Regulation EC 178/2002 defines risk as "a function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard." For some areas, different definitions have been established in legislation or by relevant international authorities. Although most definitions of "risk" have a common core (probability of adverse effects resulting from. . .), they differ in various disciplines (e.g., epidemiology, economics, sociology, toxicology).

Risk Analysis This term is not well defined in toxicology. Some consider it as a process consisting of three components: risk assessment, risk management, and risk communication.

Risk Assessment (Human Health) The evaluation of scientific information on the hazardous properties of environmental agents (hazard characterization), the dose-

response relationship (dose-response assessment), and the extent of human exposure to those agents (exposure assessment). The product of the risk assessment is a statement regarding the probability that populations or individuals so exposed will be harmed and to what degree (risk characterization).

Risk Characterization The integration of information on hazard, exposure, and dose response to provide an estimate of the likelihood that any of the identified adverse effects will occur in exposed people. The *Codex Alimentarius* definition: "The qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment."

Risk Communication An interactive process of exchanging information and views on risks between scientific experts, risk managers (at federal or state agencies), consumers, and the interested public.

Risk Evaluation Establishment of a qualitative or quantitative relationship between risks and benefits of exposure to an agent, involving the complex process of determining the significance of the identified hazards and estimated risks to the system concerned or affected by the exposure as well as the significance of the benefits brought about by the agent. It is an element of risk management. Risk evaluation is synonymous with risk-benefit evaluation.

Risk Management (Human Health) A decision-making process that accounts for political, social, economic, and engineering implications together with risk-related information in order to develop, analyze, and compare management options and select the appropriate managerial response to a potential chronic health hazard.

Risk Management Measures (RMMs) Measures in the control strategy for a hazardous substance that reduce the emission of and exposure to a substance, thereby reducing the risk to human health and the environment.

Risk Perception An integral part of risk evaluation. The subjective perception of the gravity or importance of the risk based on an individual's knowledge of different risks and the moral and political judgment attached to them and their importance.

Safety Practical certainty that adverse effects will not result from exposure to an agent under defined circumstances. It is the reciprocal of risk. Toxicological "safety" is defined as a high probability that adverse effects will not result from exposure to a chemical under specific conditions of quantity and manner of use.

Safety Factor Composite (reductive) factor by which an observed or estimated NOAEL is divided to arrive at a criterion or standard that is considered safe or without appreciable risk (see also Assessment Factor and Uncertainty Factor).

SAR Structure-activity relationship.

SCOEL The Scientific Committee for Occupational Exposure Limit values with the mandate to advise the European Commission on occupational exposure limits for chemicals at the workplace.

Sensibility An acute perception or responsiveness of an individual toward something, such as the emotions of another person or to environmental factors, e.g., noise or chemicals.

Sensitivity Personal susceptibility of an individuum to a stimulus. The concept "multiple chemical sensitivity" assumes that some individuals are more susceptible to low-dose toxic effects of chemicals than others.

Sensitivity and Specificity Statistical terms used to describe the ability of a test to correctly identify true positives and negatives. For instance, in medical diagnostics, test sensitivity is the ability of a test to correctly identify those with the disease (true positive rate), whereas test specificity is the ability of the test to correctly identify those without the disease (true negative rate).

Short-Term Exposure Limits Acronym: STEL. For a number of workplace chemicals, peak exposure or ceiling limits for short-term exposure are set, in addition to the 8-h time-weighted average (TWA) exposure limits (MAK or TLV values). According to STEL, exposure should not be longer than 15 min and should not be repeated more than four times per day. The "excursion" factor (>1) by which the STEL can exceed the TWA depends upon the chemical concerned and takes into account certain properties such as irritation.

Smog A word coined for air pollution from smoke and fog. Smog is a problem in many cities, aggravated by inversion weather conditions (which traps pollution close to the ground), and continues to harm human health. When limit values for smog are reached, warnings are given to the public.

SMR Standardized mortality ratio. This is the relative measure of the difference in risk between the exposed and unexposed populations in a cohort study. The SMR is similar to the relative risk in both definition and interpretation. This measure is usually standardized to control for any differences in age, sex, and/or race between the exposed and reference populations. It is frequently converted to a percent by multiplying the ratio by 100 (see also Mortality Rate).

Specificity The term has context-dependent meanings. In chemistry, the ability to identify and quantify the target analyte in the presence of chemically similar interfering compounds. In medicine, the ability to exclusively detect persons with a particular disease; ratio of persons with a negative test result to nondiseased persons.

Standard An environmental quality standard is the limiting concentration of a chemical (or other adverse condition, e.g., pH) permitted in a given compartment (soil, effluent, water). All standards are established for regulatory purposes and set on the basis of a judgment of a number of criteria involved: The standard is dependent on the use (e.g., drinking water or agricultural water for irrigation), the subject of protection, and the objective (protection goal).

STEL See Short-Term Exposure Limit.

Stochastic Effects The term stochastic indicates that the occurrence of effects so named would be random (with a probability <1 and >0), meaning – even for an individual – there is no threshold of dose below which the effect will not occur and the chance of experiencing the effect increases with increasing dose.

Hereditary effects and cancer induced by radiation are considered to be stochastic effects.

Stress-Strain Concept Concept in occupational medicine that describes how mechanical stress at the workplace will result in an overstraining of the musculoskeletal system. This concept is also useful for nonmechanical stress factors, such as exposures to toxicants or radioactive compounds and the resulting effects on health.

Structural Alert A molecular (sub)structure associated with the presence of a biological activity (e.g., genotoxicity).

Structure-Activity Relationship SAR: The correlation between the chemical or 3D structure of a molecule and its physicochemical properties or its biological activity. SAR analysis can help to determine chemical groups responsible for evoking a biological effect. This allows (targeted) modification of the effect or potency of a bioactive compound (e.g., a drug) by changing its chemical structure. The method has been refined to build mathematical models for the prediction of quantitative relationships between structure and biological activity (see QSAR).

Subchronic Exposure (Toxicity Study) Repeated exposure by the oral, dermal, or inhalation route for more than 30 days, up to approximately 10% of the life span in humans (more than 30 days up to approximately 90 days in typically used laboratory animal species) (see also Chronic Toxicity Study).

Subject of Protection The target object (e.g., human population, subgroup, or environment) to be protected by risk reduction measures.

Susceptibility The state or fact of being likely or liable to be influenced or harmed by a particular thing. In epidemiology or toxicology, susceptible individuals are members who are more prone to develop an illness than the (average) population at risk.

Symptoms Signs of disease. They are usually characteristic of a specific type of disease and also of a specific toxic agent.

Synergism A phenomenon in which the toxicity of a mixture of chemicals is greater than that which would be expected from the total toxicity of the individual chemicals present in the mixture (see also Combined Chemical Effects).

Systemic Toxicity Toxic effects as a result of absorption and distribution of a toxicant to a site distant from its entry point.

Target Organ The biological organ(s) most adversely affected by exposure to a chemical, physical, or biological agent.

TCCD Acronym for trichlorodibenzo[1,4]dioxin(s) or for tetrachloro-dibenzo [1,4] dioxin(s); mostly for 2,3,7,8-tetrachlorodibenzo[1,4]dioxin (see Dioxins).

TCDF Acronym for trichlorodibenzofuran(s) and for tetrachlorodibenzofuran(s) (see Dioxin).

TDI Tolerable Daily Intake; analogous to acceptable daily intake (ADI). The term "tolerable" is used for agents that are not deliberately added, such as contaminants in food.

TEF See Toxicity Equivalency Factor.

Teratogen Agent which when administered prenatally to the mother induces permanent structural malformations or defects in the offspring. The most widely known example of a teratogen is thalidomide (Contergan) which can cause severe malformations of internal organs, but mainly of extremities (limbs).

Teratogenicity Structural developmental defects due to exposure to a chemical agent during formation of individual organs.

Threshold The dose or exposure below which no deleterious effect is expected to occur.

Threshold Limit Value (TLV) Values for occupational exposure to airborne substances published by the American Conference of Governmental Industrial Hygienists (ACGIH). TLVs represent the average concentration in mg/m^3 for an 8-h workday and a 40-h work week to which nearly all workers may be repeatedly exposed, day after day, without adverse effect.

Tolerable Risk A risk which may be tolerated for transient exposure, but requires further risk reduction measures to achieve lower risk levels (see Acceptable Risk).

Toxicant An agent or material capable of producing an adverse response (effect) in a biological system, seriously injuring structure and/or function or causing death.

Toxicity Inherent property of an agent to cause an adverse biological effect. Toxicity is a general term for all undesirable or detrimental health effects of a substance and depends upon dose and properties of the substance. Based on the effect, one distinguishes between, e.g., organ toxicity, carcinogenicity, mutagenicity, embryotoxicity, and teratogenicity.

Toxicity Equivalency Factor TEF. Factor used in risk assessment to estimate the toxicity of a complex mixture, most commonly a mixture of chlorinated dibenzo-p-dioxins, furans, and biphenyls; in this case, TEF is based on relative toxicity to 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Toxicity Tests Determination of the effect of a substance on a group of selected organisms under defined conditions. A toxicity test usually measures either the proportion of organisms affected (quantal) or the degree of effect shown (graded or quantitative) after exposure to specific levels of a stimulus (concentration or dose, or mixture of chemicals) for a given period of time.

Toxicodynamics The determination and quantification of the sequence of events at the cellular and molecular levels leading to a toxic response to a chemical agent. In the context of medicinal drugs, this is referred to as pharmacodynamics.

Toxicokinetics The field of toxicology dealing with the determination and quantification of the time course of absorption, distribution, biotransformation (metabolism), and excretion of hazardous chemicals in the body. In the context of medicinal drugs, this is referred to as pharmacokinetics.

Toxicology The discipline of the adverse effects of chemical substances on living organisms. Basic toxicology characterizes the type of effects observed at different doses as well as the cellular, biochemical, and molecular mechanisms of action and the toxicokinetics. Clinical toxicology deals with the diagnosis and treatment of human and animal intoxications. Regulatory toxicology sets rules with the aim to prevent unwanted effects of chemicals.

Toxin(s) Natural poison(s); toxic organic substance(s) produced by a living organism, e.g., mycotoxins of fungi, phytotoxins of plants, or venoms of animals, often agents with partly highly specific mechanisms of action.

Toxinology The science that deals with toxins of plant, animal, and microbial origin. Toxin.

Transcriptomics Techniques available to identify the mRNA from actively transcribed genes, e.g., used to compare patterns in treated and untreated cells/organisms.

Transfer Term in environmental toxicology for the passage of a substance (e.g., cadmium) from one medium (e.g., soil) in plants.

Tumor An abnormal, uncontrolled growth of cells (synonym: neoplasm). A benign tumor is defined as a tumor that does not spread to a secondary localization, but may impair normal biological function through obstruction or may progress to malignancy later.

UBA German: Umweltbundesamt, the Federal Environmental Protection Agency.

Uncertainty Imperfect knowledge concerning the present or future state of an organism, system, or (sub)population under consideration. Lack of knowledge about variability in specific parameters in a risk assessment. Uncertainty is not the same as variability. For example, a risk assessor may be very certain that different people drink different amounts of water but may be uncertain about how much variability there is in water intakes within the population. Uncertainty can often be reduced by collecting more and better data, whereas variability is an inherent property of the population being evaluated. Variability can be better characterized with more data, but it cannot be reduced or eliminated. Efforts to clearly distinguish between variability and uncertainty are important for both risk assessment and risk characterization (see also Uncertainty Factors).

Uncertainty Factor(s) One of several, often tenfold, default factors used in operationally deriving the RfD and RfC from experimental data. The factors are intended to account for (1) variation in susceptibility among the members of the human population (i.e., interindividual or intraspecies variability); (2) uncertainty in extrapolating animal data to humans (i.e., interspecies uncertainty); (3) uncertainty in extrapolating from data obtained in a study with less-than-lifetime exposure (i.e., extrapolating from subchronic to chronic exposure); (4) uncertainty in extrapolating from a LOAEL rather than from a NOAEL; and (5) uncertainty associated with extrapolation when the database is incomplete.

Unit Risk The upper-bound excess lifetime cancer risk estimated to result from continuous exposure to an agent at a concentration of 1 mg/L in water, or 1 mg/m^3 in air. The interpretation of unit risk would be as follows: If unit risk $^1/_4\, 2 \times 10^{-6}$ per mg/L, then two excess cancer cases (upper bound estimate) are expected to develop per 1,000,000 people if exposed daily for a lifetime to 1 mg of the chemical per liter of drinking water.

Validation Process by which the reliability and relevance of a particular approach, method, process, or assessment is established for a defined purpose. Different parties define "reliability" as establishing the reproducibility or outcome of the

approach, method, process, or assessment over time. "Relevance" is defined as establishing the meaningfulness and usefulness of the approach, etc., for a defined purpose. Validation is a process required, e.g., for alternative test methods to replace for toxicity tests in animals.

Virtually Safe Dose The VSD, derived from the unit risk for a carcinogenic chemical, denotes the dose which could cause one additional case of cancer in 1,000,000 persons with lifetime exposure. In practical terms, VSD denotes a "safe" dose.

vPvB Acronym for "very persistent, very bioaccumulative." There are restrictions for compounds with such properties due to high concerns for the environment and human health.

Weight-of-Evidence (WoE) for Carcinogenicity A system used by the US EPA (and others) for characterizing the extent to which the available data support the hypothesis that an agent causes cancer in humans. The approach outlined in EPA's guidelines for carcinogen risk assessment (2005) considers all scientific information in determining whether and under what conditions an agent may cause cancer in humans and provides a narrative approach to characterize carcinogenicity rather than categories. Five standard weight-of-evidence descriptors are used as part of the narrative.

WHO World Health Organization. An independent organization of the United Nations (UN) with advisory functions, e.g., publication of guidance documents on hazardous chemicals in air, water, and food and recommendations on maximal levels in food commodities (see also ADI and PTWI).

Xenobiotic(s) A term for man-made (manufactured) chemical(s) not produced in nature and not normally considered a constituent component of a specified biological system. With regard to the latter, also phytochemicals (of natural origin but "foreign" for the mammalian organism) can be considered as xenobiotics for humans.

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