Drug Delivery Systems for Tuberculosis Prevention and Treatment

ADVANCES IN PHARMACEUTICAL TECHNOLOGY

A Wiley Book Series

Titles in the Series

Hot-Melt Extrusion: Pharmaceutical Applications
Edited by Dionysios Douroumis

Drug Delivery Strategies for Poorly Water-Soluble Drugs
Edited by Dionysios Douroumis and Alfred Fahr

Computational Pharmaceutics: Application of Molecular Modeling in Drug Delivery
Edited by Defang Ouyang and Sean C. Smith

Pulmonary Drug Delivery: Advances and Challenges
Edited by Ali Nokhodchi and Gary P. Martin

Novel Delivery Systems for Transdermal and Intradermal Drug Delivery
Edited by Ryan Donnelly and Raj Singh

Forthcoming titles:

In Vitro Drug Release Testing of Special Dosage Forms
Edited by Nikoletta Fotaki and Sandra Klein

Drug Delivery Systems for Tuberculosis Prevention and Treatment

Editor

ANTHONY J. HICKEY
RTI International, Research Triangle Park, NC, USA

Consulting Editors

AMIT MISRA
CSIR Central Drug Research Institute, Lucknow, India

P. BERNARD FOURIE
University of Pretoria, Pretoria, South Africa

WILEY

This edition first published 2016

Registered Office
John Wiley & Sons, Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, United Kingdom

Library of Congress Cataloging-in-Publication data applied for

ISBN: 9781118943175

A catalogue record for this book is available from the British Library.

Set in 10/12pt Times by SPi Global, Pondicherry, India

10 9 8 7 6 5 4 3 2 1

Contents

List of Contributors

Atul Kumar Agrawal, CSIR Central Drug Research Institute, Lucknow, India

Eusondia Arnett, Department of Microbial Infection and Immunity, Center for Microbial Interface Biology, The Ohio State University, Columbus, OH, USA

Miriam Braunstein, Department of Microbiology and Immunology, School of Medicine, University of North Carolina at Chapel Hill, NC, USA

Francesca Buttini, Department of Pharmacy, University of Parma, Viale delle Scienze, Parma, Italy *and* Institute of Pharmaceutical Science, King's College London, London, UK

Hak-Kim Chan, Advanced Drug Delivery Group, Faculty of Pharmacy, University of Sydney, Sydney, New South Wales, Australia

Gaia Colombo, Department of Life Sciences and Biotechnology, University of Ferrara, Via Fossato di Mortara, Ferrara, Italy

Shyamal C. Das, School of Pharmacy, University of Otago, Dunedin, New Zealand

Robert DeLong, Department of Anatomy and Physiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA

P. Bernard Fourie, Department of Medical Microbiology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

Lucila Garcia-Contreras, College of Pharmacy, The University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

Stefano Giovagnoli, Department of Pharmaceutical Sciences, University of Perugia, Perugia, Italy

Mercedes Gonzalez-Juarrero, Mycobacteria Research Laboratories, Microbiology Immunology and Pathology, Colorado State University, Fort Collins, CO, USA

Anuradha Gupta, CSIR Central Drug Research Institute, Lucknow, India

Richard Hafner, National Institute of Allergy and Infectious Disease *and* National Institutes of Health, Bethesda, MD, USA

Graham F. Hatfull, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA

David L. Hava, Pulmatrix Inc., Lexington, MA, USA

Anthony J. Hickey, Discovery Sciences Technologies Group, RTI International, Research Triangle Park, NC, USA

Mary K. Hondalus, Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, GA, USA

Mariam Ibrahim, College of Pharmacy, The University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

Nitya Krishnan, MRC Centre for Molecular Bacteriology and Infection, Department of Medicine, Imperial College London, UK

Nitesh K. Kunda, Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico, Albuquerque, NM, USA

Andre G. Loxton, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

Kimiko Makino, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Japan

Amber A. McBride, Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico, Albuquerque, NM, USA

David N. McMurray, Department of Microbial Pathogenesis and Immunology, College of Medicine, Texas A&M University Health Science Center, College Station, TX, USA

Amit Misra, CSIR Central Drug Research Institute, Lucknow, India

Pavan Muttil, Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico, Albuquerque, NM, USA

Edward A. Nardell, Division of Global Health Equity, Brigham and Women's Hospital, Boston, MA, USA

Ruvandhi R. Nathavitharana, Division of Infectious Diseases, Beth Israel Deaconess Medical Center, Boston, MA, USA

Sanketkumar Pandya, CSIR Central Drug Research Institute, Lucknow, India

Dominique N. Price, Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico, Albuquerque, NM, USA

Rajeev Ranjan, CSIR Central Drug Research Institute, Lucknow, India

Maurizio Ricci, Dipartimento di Chimica e Tecnologia del Farmaco, Università degli Studi di Perugia, Perugia, Italy

Brian D. Robertson, MRC Centre for Molecular Bacteriology and Infection, Department of Medicine, Imperial College London, London, UK

Carlo Rossi, Department of Pharmaceutical Sciences, University of Perugia, Perugia, Italy

Madhur Sachan, CSIR Central Drug Research Institute, Lucknow, India

Samantha L. Sampson, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

Larry S. Schlesinger, Department of Microbial Infection and Immunity, Center for Microbial Interface Biology, The Ohio State University, Columbus, OH, USA

Aurélie Schoubben, Department of Pharmaceutical Sciences, University of Perugia, Perugia, Italy

Peter J. Stewart, Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia

Jean C. Sung, Pulmatrix Inc., Lexington, MA, USA

Hiroshi Terada, Niigata University of Pharmacy and Applied Sciences, Niigata, Japan

Reinhard Vehring, Department of Mechanical Engineering, University of Alberta, Edmonton, Alberta, Canada

Jennifer Wong, Advanced Drug Delivery Group, Faculty of Pharmacy, University of Sydney, Sydney, New South Wales, Australia

Ellen F. Young, Department of Microbiology and Immunology, School of Medicine, University of North Carolina at Chapel Hill, NC, USA

Foreword

"As a physician, I have seen how much pain TB patients experience after months of treatment by intramuscular injection (IM). It is almost impossible to inject by IM after one month. I think that aerosol delivery is the future for TB drug delivery because it is directly delivered to the target organ, and it is even more important for patients who have a hard time to take pills. I believe that aerosol delivery of TB drug(s) will be a milestone in TB treatment if successful." Li Liang, Vice Director Beijing Chest Hospital

Having plagued societies for centuries, tuberculosis (TB) is one of the oldest diseases known to man. While the first drug effective against TB was not developed until 1943, over the next three decades many additional anti-TB drugs were discovered and developed that significantly reduced morbidity and mortality. Yet today it is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis*. The most recent World Health Organization's report indicated that TB killed 1.5 million people in 2014, making it a larger cause of death than HIV/AIDS, which was responsible for 1.2 million deaths. Thus, despite the perception that tuberculosis is a disease of the past or a disease of only low-income countries, it remains a major global public health challenge that carries significant global and domestic disease burdens and risks. Because serious societal challenges remain, including extreme poverty, inequity, and disproportionate TB burdens in women and children, TB will remain a significant challenge for the foreseeable future. Furthermore, the face of TB is changing. While global numbers of new TB cases and TB deaths have decreased at an average rate of at least 2 percent per year, TB strains that are resistant to the most commonly used, inexpensive, and least-toxic TB drugs have been identified in almost every country. These *multidrug-resistant TB* (MDR-TB) strains as well as the growing numbers of the even more serious *extensively drug-resistant TB* (XDR-TB) strains have been reported from nearly all countries. MDR-TB and XDR-TB cases can be exceedingly difficult and expensive to diagnose and treat successfully.

One of the major barriers to treatment of MDR-TB today is the high cost of second-line drugs that may be 300 to 3000 times more expensive than first-line therapy. Second-line regimens which are administered for between 18 to 24 months are associated with significant adverse events that often lead to discontinuation of treatment. Despite prolonged treatment duration, these regimens are not associated with high cure rates and incomplete, sub-optimal therapy of MDR-TB likely contributes to emergence of XDR-TB. In the face of *M. tuberculosis* strains resistant to all known classes of anti-TB drugs, leaders in global public health are asking whether XDR-TB is signaling a return to a pre-antibiotic era in TB control. Thus the need for new TB drugs has never been more urgent. Importantly, the search for new regimens and alternative strategies requires a thorough understanding of the preparation and performance of dosage forms.

Recent important gains in TB discovery research, product development, and implementation science and regulatory approval of the first new TB drug in 30 years give reason for optimism. Systematic studies of the biological effects of TB infection are beginning to shed light on the complexity of the human immune response and the dynamic nature of the disease process. As the disease becomes better understood in terms of both pathogen and host molecular biology there is an opportunity for new pharmaceutical approaches based on the route and means of delivery of a range of novel therapeutic agents. New studies are identifying molecules that can be used to diagnose TB or provide the basis of new TB vaccine research strategies, as well as critical biological processes against which new drug targets can be identified. Indeed the current global TB pipeline has multiple candidates in clinical trials – but there are few novel molecular entities. Many more candidates with novel mechanisms of action and chemical diversity are needed to overcome historical drug development attrition rates and emergence of resistance.

In the past, natural products have played a pivotal role in antibiotic drug discovery with most antibacterial drugs being derived from a natural product or natural product lead. A key challenge in the development of natural products as drugs is to combine their inherent antibacterial properties with physicochemical properties that confer oral bioavailability, an attribute that is highly desirable for treatment of MDR-TB. Many drugs are lost to development due to lack of oral bioavailability. However, new approaches to TB drug delivery as described in the current volume have the potential to overcome this barrier. New developments in drug delivery systems and technologies open an exciting avenue that may potentially lead to the repurposing of old drugs and re-evaluation of potential new drugs hitherto thought undeliverable.

Finally, while BCG vaccine remains the world's most widely used vaccine and protects children against disseminated TB and meningitis, its effectiveness in preventing disease in adults varies widely. New candidate vaccines are being developed that provide protection against disease and possibly infection in animal models. Since the battle between the pathogen and immune response in TB is fought out largely in the lung, it will be essential both to understand protective immune responses in the lung and how to deliver new vaccine candidates to generate protection in the lung. This is another of the key issues in TB treated in this book.

This is a timely volume addressing the application of pharmaceutical sciences and dosage-form design to the development of novel strategies for TB therapy. This volume is arranged to consider the nature of disease, immunological responses, vaccine and drug

delivery, disposition and response. In addition to conventional treatments some novel approaches are presented that if successful would create rapid development pathways. The contributors are drawn from the relevant fields of microbiology, immunology, molecular biology, pharmaceutics, pharmacokinetics, and chemical and mechanical engineering. No doubt the knowledge shared by the authors will have a major impact upon development of urgently needed new tools to address the continuing global crisis of TB and the increasing threat of drug-resistant strains.

Gail H. Cassell, Ph.D., D.Sc. (hon)
Executive Vice President TB Drug Development
Infectious Disease Research Institute
Senior Lecturer, Department of Global Health and Social Medicine
Harvard Medical School
Vice President Scientific Affairs, Eli Lilly and Company (ret.)

Barbara E. Laughon, Ph.D.
Senior Scientist for TB Drug Development Partnerships in the Office of the Director
Division of Microbiology and Infectious Diseases at the National Institute of Allergy and Infectious Diseases
U.S. National Institutes of Health

Barry Bloom, Ph.D.
Harvard University Distinguished Service Professor and Joan L. and Julius H. Jacobson Professor of Public Health
Harvard T. H. Chan School of Public Health

Li Liang, M.D.
Vice Director, Beijing Chest Hospital
Vice Director, Clinical Center on Tuberculosis, China CDC
Designated Director General, TB Society of China Medical Association

Advances in Pharmaceutical Technology: Series Preface

The series *Advances in Pharmaceutical Technology* covers the principles, methods and technologies that the pharmaceutical industry uses to turn a candidate molecule or new chemical entity into a final drug form and hence a new medicine. The series will explore means of optimizing the therapeutic performance of a drug molecule by designing and manufacturing the best and most innovative of new formulations. The processes associated with the testing of new drugs, the key steps involved in the clinical trials process and the most recent approaches utilized in the manufacture of new medicinal products will all be reported. The focus of the series will very much be on new and emerging technologies and the latest methods used in the drug-development process.

The topics covered by the series include the following:

Formulation: The manufacture of tablets in all forms (caplets, dispersible, fast-melting) will be described, as will capsules, suppositories, solutions, suspensions and emulsions, aerosols and sprays, injections, powders, ointments and creams, sustained release and the latest transdermal products. The developments in engineering associated with fluid, powder and solids handling, solubility enhancement and colloidal systems, including the stability of emulsions and suspensions, will also be reported within the series. The influence of formulation design on the bioavailability of a drug will be discussed and the importance of formulation with respect to the development of an optimal final new medicinal product will be clearly illustrated.

Drug Delivery: The use of various excipients and their role in drug delivery will be reviewed. Amongst the topics to be reported and discussed will be a critical appraisal of the current range of modified-release dosage forms currently in use and also those under development. The design and mechanism(s) of controlled-release systems including macromolecular drug delivery, microparticulate-controlled drug delivery, the delivery of biopharmaceuticals, delivery vehicles created for gastrointestinal tract-targeted delivery, transdermal delivery and systems designed specifically for drug delivery to the lung will

all be reviewed and critically appraised. Further site-specific systems used for the delivery of drugs across the blood–brain barrier including dendrimers, hydrogels and new innovative biomaterials will be reported.

Manufacturing: The key elements of the manufacturing steps involved in the production of new medicines will be explored in this series. The importance of crystallization; batch and continuous processing; seeding; and mixing including a description of the key engineering principles relevant to the manufacture of new medicines will all be reviewed and reported. The fundamental processes of quality control including good laboratory practice, good manufacturing practice, Quality by Design, the Deming Cycle, Regulatory requirements and the design of appropriate robust statistical sampling procedures for the control of raw materials will all be an integral part of this book series.

An evaluation of the current analytical methods used to determine drug stability, as well as the quantitative identification of impurities, contaminants and adulterants in pharmaceutical materials will be described, as will the production of therapeutic bio-macromolecules, bacteria, viruses, yeasts, moulds, prions and toxins through chemical synthesis and emerging synthetic/molecular biology techniques. The importance of packaging including the compatibility of materials in contact with drug products and their barrier properties will also be explored.

Advances in Pharmaceutical Technology is intended as a comprehensive one-stop shop for those interested in the development and manufacture of new medicines. The series will appeal to those working in the pharmaceutical and related industries, both large and small, and will also be valuable to those who are studying and learning about the drug-development process and the translation of those drugs into new life-saving and life-enriching medicines.

Dennis Douroumis
Alfred Fahr
Jűrgen Siepmann
Martin Snowden
Vladimir Torchilin

Preface

Tuberculosis remains the world's most serious cause of disease due to a single infectious micro-organism. Despite the development of a vaccine almost a century ago and with the advent of drug treatment in the intervening period we appear to be no closer to eradicating this disease. New vaccine antigens and novel drugs have been the major focus in prevention and treatment of tuberculosis. While great effort has been expended and progress has been made in drug therapy it has occurred at a remarkably slow pace. Indeed, the challenges posed by multiple and extensively drug-resistant disease and co-infection with human immuno-deficiency virus have rendered the need for novel approaches urgent.

As the disease becomes better understood in terms of both pathogen and host molecular biology there is an opportunity for new pharmaceutical approaches based on the route and means of delivery of a range of novel therapeutic agents.

This volume is arranged to consider the nature of disease, immunological responses, vaccine and drug delivery, disposition and response. In addition to conventional treatments some novel approaches are presented that, if successful, would create rapid development pathways. The contributors are drawn from the relevant fields of microbiology, immunology, molecular biology, pharmaceutics, pharmacokinetics, and chemical and mechanical engineering.

The role of therapeutic targeting strategy, dosage-form design and route of administration in the effective treatment of tuberculosis has been a topic of personal interest that we have shared for approaching twenty years and it is our privilege to be able to bring current thinking on a range of topics into one volume. We owe a great deal to our friends and colleagues most of whom are authors of chapters in this volume who attended the meetings on 'Inhaled Tuberculosis Therapy' held in New Delhi and Tokyo in 2009 and 2013, respectively. Without their insight, enthusiasm and encouragement we would not have been able to complete this text.

It has been a great pleasure working with the staff at Wiley on the preparation of the book and we are particularly grateful for the contributions of Samanaa Srinivas, Emma Strickland and Rebecca Stubbs. Many thanks for their patience and accommodation throughout the process.

Anthony J. Hickey,
Research Triangle Park, NC, USA

Amit Misra,
Lucknow, India

P. Bernard Fourie,
Pretoria, South Africa

July 2016

1

Introduction: A Guide to Treatment and Prevention of Tuberculosis Based on Principles of Dosage Form Design and Delivery

A.J. Hickey

RTI International, RTP, NC, USA

1.1 Background

Tuberculosis has been a scourge of mankind for millennia. The discovery by Koch of the causative organism *Mycobacterium tuberculosis* at the end of the nineteenth century was hailed as the discovery that would rapidly lead to its eradication [1]. Despite the speed of development of a vaccine, attenuated *Mycobacterium bovis* (bacille Calmette Guerin), and the discovery of a therapeutic drug within only a few decades, circumstances that could not have been foreseen with respect to new strains, multiple-drug resistance and co-infection with human immunodeficiency virus, have rendered the disease a more complicated challenge than originally envisaged.

As the twentieth century progressed physicians were horrified to discover that the vaccine was not universally protective and that resistance to the drug of choice, streptomycin, was increasing rapidly [2]. These observations led to further activities in both the realm of vaccine and drug development, the latter being the more clinically successful but the former yielding much need information on the pathogen, the host immunity and pathogenesis of disease.

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

During this period pharmacy and pharmaceutical dosage form design were also entering a golden age. Manufacturing of drug products or compounding, which was traditionally an activity that took place in a pharmacy, was transferred to an industrial setting. Commercial products involving a variety of dosage form were being standardized to allow production on a scale previously unknown. The introduction of legislation regulating the quality of products, particularly to address adulteration and ensure safety, commenced most notably in the 1930s with the Food Drug and Cosmetics Act of the United States [3]. In the latter half of the twentieth century the underlying physical chemistry and chemical engineering required to manufacture under rigorously controlled conditions that ensured the quality, uniformity, efficacy and safety of the product were developed.

With this background it is noteworthy that the parallel developments in dosage form and tuberculosis (TB) treatment led to their convergence in the early part of the twentieth century when reproducible drug delivery could only be achieved by oral administration (tablets and capsules) or parenteral administration (injection). As a consequence, other routes and means of delivery were rarely, if ever, considered for the delivery of drugs or vaccines. This can be contrasted with the products of biotechnology developed in the late twentieth century for which both oral and parenteral administration were rarely feasible. Of course, the ease of delivery and the required dose were the leading reasons for the selection of these routes of administration.

There was a brief period in the middle of the twentieth century when the absence of new drugs and the increase in drug resistance led to studies of inhaled therapy for tuberculosis but the development of new drugs resulted in this approach being abandoned and only revisited during times when there were no apparent oral and parenteral dosage forms to meet the immediate challenge. Figure 1.1 presents the number of publications that can be found in the accessible literature for the period since the initial rise in drug-resistant tuberculosis in the 1940s. A subsequent peak appears following the rise in human immunodeficiency virus co-infected patients and multiple-drug-resistant tuberculosis requiring alternative therapeutic strategies.

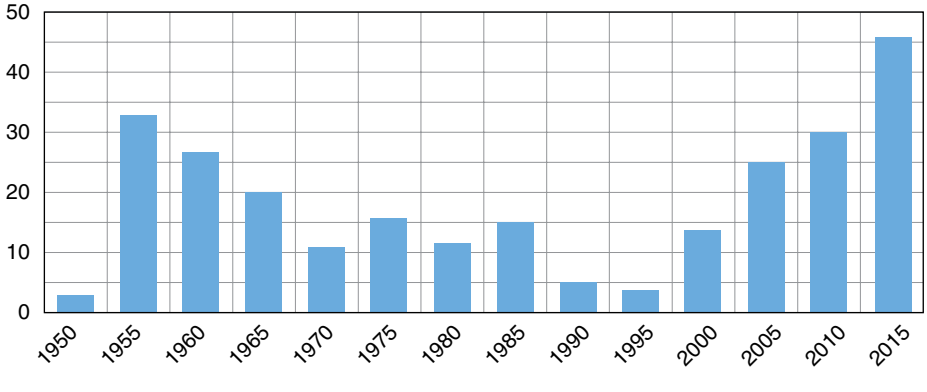


Figure 1.1 *Reports of Aerosol Delivery Extracted from PubMed from the earliest citations in the modern literature*

1.2 Dosage Form Classification

The route of administration by which drugs are delivered dictates the dosage form employed. The United States Pharmacopeia has classified therapeutic products in terms of three tiers: route of administration, dosage forms and performance test which captures all conventional and most novel strategies for disease treatment as shown in Figure 1.2 [4]. The performance measure of significance for the majority of dosage forms is the dissolution rate which, together with the biological parameter of permeability for those drugs presented at mucosal sites, dictates the appearance of the drug in the systemic circulation and ultimately its therapeutic effect.

1.2.1 Dosage Forms

It would not be possible to do justice to the science and technology underpinning the wide range of dosage forms available for drug delivery. However, to put those used in the treatment and prevention of tuberculosis in context a brief review of the key components and processes involved may be helpful to the reader.

1.2.1.1 Solid Oral Dosage Forms

These consist of a mixture of powders each of which is intended to confer a desirable property on the dosage form that leads to effective manufacture, drug delivery and therapeutic effect [5, 6].

In addition to the drug substance which must be well characterized, glidants help the powder flow which aids in filling, surfactants enhance dissolution and diluents are considered inert bulking agents that assist in metering small quantities of drug during filling and may help in compaction. Binding agents, as the name suggests, help in binding all components into a granule or tablet to preserve the integrity of the dosage form on storage and

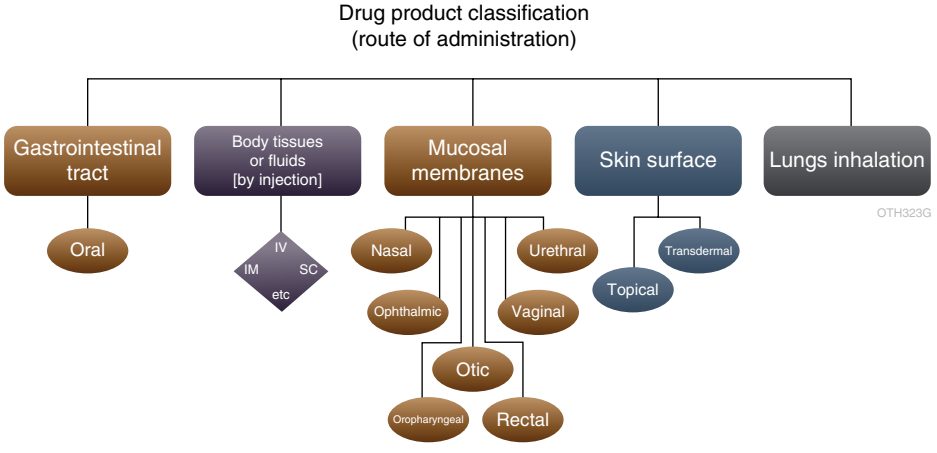


Figure 1.2 *United States Pharmacopeia Taxonomy of Dosage Forms structured from: Tier 1 – Route of Administration; through Tier 2 – Dosage Form to; Tier 3 – Performance (not shown). (Modified from ref. [4] Courtesy of Margareth Marques and the USP)*

prior to administration. The common dosage forms are capsules and tablets that differ in that the former consists of a powder or granulated loose fill while the latter requires compaction [5, 6]. The most common capsule is prepared with gelatin and filled with the optimized formulation of drug in excipients to allow for stability on storage and reproducible and efficacious dose delivery. Tablets also contain the drug and excipient compacted into a single solid dosage form that has desired performance properties in terms of stability, dissolution, dose delivery and efficacy. Biopharmaceutical considerations are of great significance to the disposition of drugs from solid oral dosage forms. Their behavior under the wide range of pH conditions (1–8) in the gastro-intestinal tract and an understanding of the influence of anatomy and physiology on local residence time and regions of absorption are significant considerations in optimization of the dosage form. Relatively recently the publication of Lipinski's rules [7] and the biopharmaceutical classification system [8] have been an enormous help in the selection of drugs and requirements of formulations that correlate with successful drug delivery by the oral route of administration.

1.2.1.2 Parenteral Dosage Forms

These are intended for injection either directly into the blood circulation [intravenous (IV)] or at a site from which the drug can readily be transported to the vasculature as would occur following subcutaneous or intramuscular administration [9]. There are other infrequently employed (intraperitoneal) or specialized (intrathecal or intratumoral) sites of injection that are not relevant to tuberculosis therapy. The key elements of a parenteral dosage form are the requirement for a formulation suitable for delivery from a syringe through a needle to the intended site. The formulation can range from simple solutions to a variety of dispersed systems (emulsions, micelles, liposomes and solid suspensions). Important physico-chemical properties must be considered to avoid local tissue damage on injection. Primarily these relate to the requirement to approximate physiological pH and ionic strength (tonicity) [10]. However, there are other safety considerations for injectable dispersed systems that relate to physical obstruction of capillaries (embolism), as well as uptake by the reticulo-endothelial system (inflammation, irritation or immune responses) [11]. The composition of any excipients, carrier systems and the nature of the injected active ingredient will dictate expectations of any of these responses.

1.2.1.3 Inhaled Dosage Forms

These deliver droplets or particles to the pulmonary mucosa that are then distributed locally and transported to the systemic circulation by absorption. The most important criteria for the efficacy of inhaled therapeutics are the aerodynamic particle size distribution and the dose delivered. The particle size range that is targeted for efficient delivery of drug to the lungs is 1–5 μm [12]. The United States Pharmacopeia has described types of inhaled drug product. Of those shown in Figure 1.3 the most important aerosol products for the treatment of pulmonary disease fall into three categories: metered dose inhalers (MDIs), dry powder inhalers (DPIs), and nebulizer systems. MDIs employ high-vapor-pressure propellant to deliver rapidly evaporating droplets containing the active ingredient; dry powder inhalers deliver particles of drug alone or by the use of a carrier particle; and nebulizers deliver aqueous solutions or suspensions of the active ingredient [12]. It is important to note that the primary performance measures for aerosol systems are aerodynamic particle size distribution and delivered dose since these are determinants of the drug reaching the

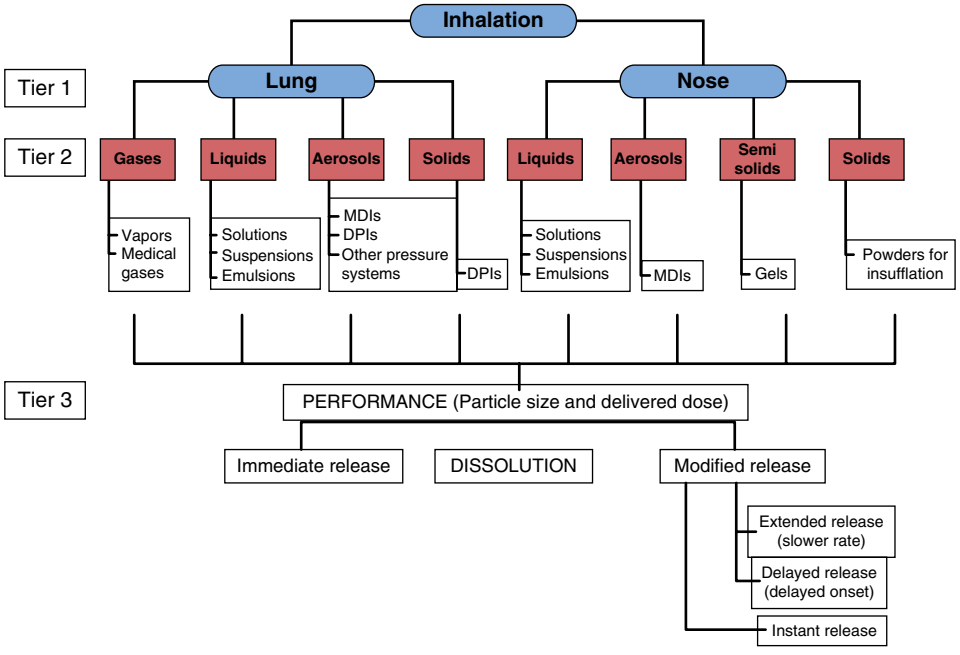


Figure 1.3 *Dosage forms intended for delivery of drugs to the respiratory tract divided according to the USP taxonomy of route of administration (tier 1), dosage form (tier 2) and performance measures (tier 3)(Modified from Ref. [4] Courtesy of Vinod Shah and the USP)*

mucosal site for action or absorption. Owing to the solubility, very small particle size and surface area of inhaled particles and droplets, dissolution is rarely the dominant factor in drug bioavailability. However, where the drug substance exhibits poor solubility or is prepared as a controlled release, dissolution is limited, and formulation dissolution rate will play an important role in location and extent of bioavailability.

Metered dose inhaler formulations are non-aqueous-based solutions or suspensions and in general are limited to delivering boluses of relatively low doses, rarely above a milligram. Dry powder inhalers in which carriers such as lactose particles are employed also deliver boluses of relatively low doses. However, the use of drug alone in engineered particles has increased the potential dose to 100 mg. Nebulizers do not deliver bolus doses, rather they deliver steady-state aerosols from a reservoir until the fixed volume has been depleted. The total dose delivered from these devices is only limited by the rate (liquid volume/time) and duration of delivery. Delivery for 15–20 minutes is commonly conducted, and precedent for the dose of antimicrobial agent has been set at several hundred milligrams.

1.3 Controlled and Targeted Delivery

In the mid-1980s the attention of some researchers turned to controlling the dissolution rate of orally administered drugs to treat tuberculosis by preparing polymeric microparticles [13, 14]. The intent was to more effectively deliver the drug and to potentially increase the duration of action by extending the period that circulating concentrations remained above

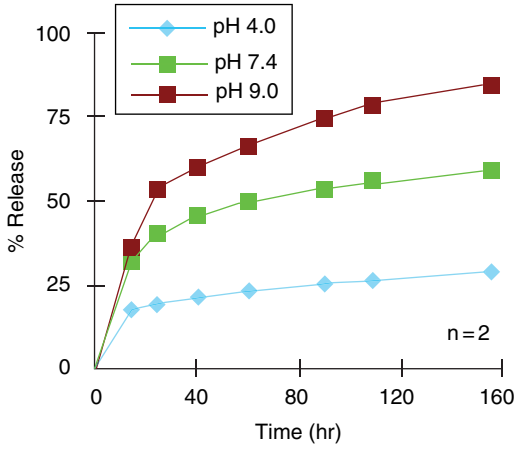


Figure 1.4 *Dissolution of 7.5% rifampicin in poly(lactide-co-glycolide) in three media of different pH values (4.0, 7.4 and 9.0) (Ref. [15])*

the minimum inhibitory concentration. Interestingly, when the dissolution profiles of rifampicin are examined as shown in Figure 1.4, the effect of pH, in the range of relevance to oral delivery, is to lower the dissolution rate and extent at lower pH. This raised the potential not only for controlled but also targeted delivery when particles of similar composition but in a respirable size range were delivered by inhalation. Aerosol particles that do not dissolve immediately when delivered to the lungs are phagocytosed by alveolar macrophages and the low pH (~5.0–5.5) in the endosome presents the opportunity for extended duration of delivery [15]. Therefore, the therapeutic effect will be enhanced in this location within the host cell for *Mycobacterium tuberculosis* [16, 17]. This observation has since launched a wide range of control and targeting strategies (nanoparticles, liposomes, micelles, etc.) for drug delivery to the lungs to treat tuberculosis [18]. The link to observations from oral delivery should not be forgotten. As more potent agents are developed and gastro-intestinal targeting strategies are informed by greater biological and biophysical understanding it is conceivable that lessons from pulmonary delivery can be translated into future options for oral dosage forms.

1.4 Physiological and Disease Considerations

Delivery of drugs by the oral route in tablets or capsules requires that the drug is absorbed and distributed from the gastro-intestinal tract to the systemic circulation where it can subsequently present to infected organs and tissues at concentrations sufficient (above the minimum inhibitory concentration) to treat the infection. The large volume of distribution for systemically circulating drugs currently in use for TB therapy usually requires large amounts of drug in order to achieve therapeutic concentrations. The need for multiple drug therapy for many months is a burden for patients and is seriously exacerbated in those with multiple or extensively drug-resistant disease where many more drugs are administered for even longer periods of time. Simply ingesting the large quantities of medicine required is

an ordeal. However, in principle oral delivery remains the simplest means of administration, the least invasive and most convenient approach for the patient, and requires no special storage or disposal requirements.

Parenteral administration by whichever route (commonly subcutaneous, intramuscular, intravenous) ensures the delivery of a controlled dose and as an invasive method circumvents the need for absorption by placing the drug either in or near the circulatory system. However, this approach is quite often painful for the patient and has additional storage and hazardous waste disposal requirements that are not required for other dosage forms.

Tuberculosis is contracted by pulmonary deposition of virulent organisms and the subsequent proliferation of disease from the lungs. The majority of individuals develop natural immunity that clearly originates at the pulmonary mucosa. Consequently, it is reasonable to propose that presentation of vaccines or drugs to this site will offer an advantage in disease prevention or therapy. Inhaled therapy has been well established through the administration of drugs to treat asthma and chronic obstructive pulmonary disease (COPD). More recently, the interest in treating other pulmonary infectious diseases has resulted in the approval of tobramycin to treat *Pseudomonas aeruginosa* in cases of cystic fibrosis [19]. Therefore, the precedent has been set for the delivery of doses of drug sufficient to treat local infection.

1.5 Therapeutic Considerations

When considering a route of administration several practical questions must be considered:

1. What is the target?
2. What dose is required for therapeutic effect?
3. What is the maximum tolerated or feasible dose?
4. Are there off-target effects?
5. Are there any drug interactions?
6. Are there any metabolic considerations?
7. Are there drug specific physico-chemical property limitations or advantages?

While there are many means and routes of administration it is generally accepted that for those drugs that are orally bioavailable following ingestion into the gastro-intestinal tract, tablets and capsules are a desirable dosage form. However, not every drug, disease and indication lends itself to oral delivery.

The diversity of geographical locations in which tuberculosis occurs does not support every route of administration equally under all circumstances. It is particularly notable that parenteral products require needles, syringes and, often, cold chain for transport and storage. These requirements add an additional level of complexity in distribution and maintenance of an adequate supply in remote or impoverished locations.

The advent of multiple and extensively drug-resistant disease and the conundrum of treatment that might be effective against latent or persistent disease has been the cause for exploring other routes and means of delivery of drugs, the most notable of which is aerosol therapy to the lungs.

In order to understand the role of the dosage form in effective disease treatment and prevention a range of considerations must be explored. The purpose of this volume is to

examine the pathogenesis of disease, animal models required to adequately assess new approaches, conventional and novel methods of preparing drugs and vaccines for delivery, testing strategies to evaluate the impact of any strategy, new considerations that might complement or disrupt the traditional approach to therapy (immunotherapeutics, biofilm busters, phage therapy) and finally anticipated clinical strategies. This will then serve the purpose of giving those involved in drug and vaccine development, dosage form design and delivery of therapeutic agents a foundation from which to consider the path to new and effective products.

1.6 Conclusion

Tuberculosis therapy and prevention has been driven by major discoveries in basic understanding of the disease, new drugs and potential new vaccines. However, the increase in multiple and extensively drug-resistant tuberculosis, HIV co-infection and absence of an approach to the treatment of latent and persistent disease still confounds our ability to control and ultimately eradicate this disease. The effectiveness of any drug or vaccine is only as good as the ability to deliver it in efficacious doses to the desired site of action which, in turn, is dictated by the nature of the dosage form, the delivery system and the disposition of the active agent following delivery. The intent of this volume is to consider the role that each of these elements plays currently, and explore future possibilities that arise from ongoing scientific and technological advances.

References

[1] Sakula, A. (1983) Robert Koch: Centenary of the discovery of the tubercle bacillus (1882), *Can. Vet. J.*, **24**, 127–131.
[2] Hickey, A.J., Durham, P.G., Dharmadhikari, A. and Nardell, E.A. (2016), Inhaled drug treatment for tuberculosis: Past Progress and Future Prospects, *J. Controlled Release*, submitted.
[3] Federal Food, Drug and Cosmetics Act (1938) To prohibit the movement in interstate commerce of adulterated and misbranded food, drugs, devices, and cosmetics, and for other purposes.
[4] Marshall, K., Foster, T.S., Carlin, H.S. and Williams, R.L. (2003) Development of a compendial taxonomy and glossary for pharmaceutical dosage forms. *Pharm. Forum*, **29**, 1741–1752.
[5] Augsburger, L.L. (2002) Hard and Soft Shell capsules, in *Modern Pharmaceutics*, 4th edn (eds G. Banker and C. Rhodes), Marcel Dekker, New York, NY, pp. 335–380.
[6] Kottke, M.J. and Rudnik, E.M. (2002) Tablet dosage forms, in *Modern Pharmaceutics*, 4th edn, Marcel Dekker, New York, NY, pp. 287–333.
[7] Lipinski, C.A., Lombardo, F., Dominy, B.W. and Feeney, P.J. (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, *Adv. Drug. Del. Rev.*, **46**, 3–26.
[8] Amidon, G.L., Lennernas, H., Shah, V.P. and Crison, J.R. (1995) A theoretical basis for a biopharmaceutical drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability, *Pharm. Res.*, **12**, 413–420.
[9] Boylan, J.J. and Nail, S.L. (2002) Parenteral Products, in *Modern Pharmaceutics*, 4th edn, Marcel Dekker, New York, NY, pp. 381–414.
[10] Martin, A. (1993) Non-electrolytes, electrolytes, ionic equilibria and buffers, in *Physical Pharmacy*, 4th edn, Lea and Febiger, Malvern, MA, pp. 101–189.
[11] Langille, S.L. (2013) Particulate matter in injectable drug products, *PDA J. Pharm. Sci. Technol.*, **67**, 186–200.

[12] Hickey, A.J. (2004) Summary of common approaches to pharmaceutical aerosol administration, in *Pharmaceutical Inhalation Aerosol Technology*, 2nd edn, (ed. A.J. Hickey), Marcel Dekker, New York, NY, pp. 385–421.
[13] Denkbas, E.B., Kaitan, X., Tuncel, A. and Piskin, E. (1995) Rifampicin-carrying poly (D,L-lactide) microspheres: Loading and release, *J. Biomater. Sci., Polym. Ed.*, **6**, 815–825.
[14] O'Hara, P., and Hickey, A.J. (2000) Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: manufacture and characterization, *Pharm. Res.*, **17**, 955–961.
[15] Barrow, E.L., Winchester, G.A., Staas, J.K., Quenelle, D.C. and Barrow, W.W (1998) Use of microsphere technology for targeted delivery of rifampicin to Mycobacterium tuberculosis-infected macrophages, *Antimicrob. Agents Chemother.*, **42**, 2682–2689.
[16] Suarez, S., O'Hara, P., Kazantseva, M., Newcomer, C.E., Hopfer, R., McMurray, D.N. and Hickey, A.J. (2001) Airways delivery of rifampicin microparticles for the treatment of tuberculosis, *J. Antimicrob. Chemother.*, **48**, 431–434.
[17] Suarez, S., O'Hara, P., Kazantseva, M., Newcomer, C.E., Hopfer, R., McMurray, D.N. and Hickey, A.J. (2001) Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: screening in an infectious disease model, *Pharm. Res.*, **18**, 1315–1319.
[18] Geller, D.E., Weers, J. and Heuerding, S. (2011) Development of an inhaled dry powder formulation of tobramycin using PulmoSphere™ technology. *J. Aerosol. Med. Pulm. Drug Deliv.*, **24**, 175–182.
[19] Mortensen, N.P., Durham, P. and Hickey, A.J. (2014) The role of particle physico-chemical properties in pulmonary drug delivery for tuberculosis therapy. *J Microencapsul.*, **31**, 785–795.

Section 1

Pathogen and Host

2

Host Pathogen Biology for Airborne *Mycobacterium tuberculosis*: Cellular and Molecular Events in the Lung

Eusondia Arnett[1,], Nitya Krishnan[2,*], Brian D. Robertson[2] and Larry S. Schlesinger[1]*

[1] Department of Microbial Infection and Immunity, Center for Microbial Interface Biology, The Ohio State University, Columbus, OH, USA

[2] MRC Centre for Molecular Bacteriology and Infection, Department of Medicine, Imperial College, London, UK

2.1 Introduction

Tuberculosis (TB), caused by the slow-growing, obligate human pathogen *Mycobacterium tuberculosis* (*M. tuberculosis*), has probably occurred in humans for a significant part of their history. Just exactly how long is a matter of debate, with differing dates suggested depending on the methodology and materials used. One study, based on whole genome sequences representing the diversity of *M. tuberculosis* across the planet [1], puts TB with humans since pre-history, some 70,000 years ago, subsequently moving with human populations out of Africa and across the globe. This would mean that TB only arrived in the Americas post-Columbus, and fits with the idea that *M. tuberculosis* first evolved to live in low-density hunter-gatherer populations where the chances of meeting a new host were low, making long-term, latent infections an evolutionary advantage. Another study [2] is based on mycobacterial DNA collected from Peruvian skeletons with evidence of TB dating from

[*] Equal contributors

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

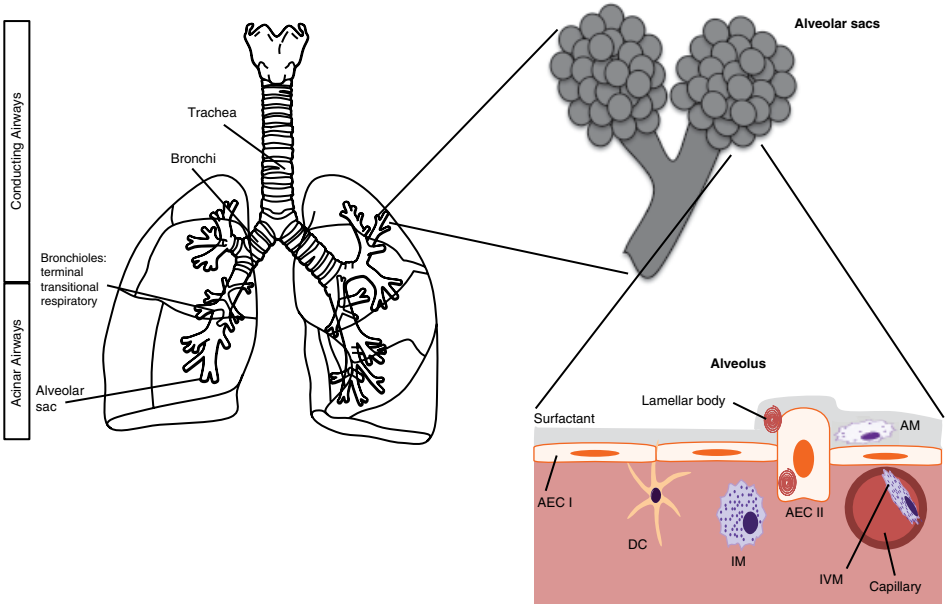
1028–1280 AD, so predating European contact. Genome analysis and two independent dating methods put the most recent common ancestor for the *M. tuberculosis* complex less than 6,000 years ago, more than a 10-fold difference compared with the Comas study [1]. The Bos phylogeny put the Peruvian strains close to *M. pinnipedii*, implicating a role for sea mammals transmitting the disease to humans across the ocean [2]. Which estimate is correct, or whether both can be reconciled, will require further archaeological material and confirmatory data.

Regardless of when TB arose in humans, it is a problem that remains with us into the 21st century, despite all of the scientific, technological and medical advances that have been made. Current WHO data for 2012 report 9 million new cases of diagnosed disease and 1.5 million deaths, one person every 25 seconds – truly a sobering figure in the 21st century. There is an effective drug therapy available to treat those patients, but it requires 3 or 4 drugs for at least 6 months, making compliance poor and effective maintenance of the drug supply difficult for a disease of low- and middle-income countries, often with poor public health infra-structure. Drug-resistant organisms (MDR-TB) are an increasing problem, with some infections (XDR-TB) untreatable. However, even if we could successfully treat and cure those 9 million cases, there is, like an iceberg, a much bigger problem lying underneath. Based on immunological reactivity and epidemiology there are an estimated 2 billion people carrying a sub-clinical or latent infection with *M. tuberculosis*, each with a variable risk of the infection reactivating to become clinically significant with transmissible bacteria. We currently have no means of identifying who those patients are, no way to stratify their risk of reactivating infection, and no specific treatment. This highlights several key areas for the control of tuberculosis. We need new drugs to shorten treatment, tackle drug-resistant organisms, and target sub-clinical infections. To make optimal use of both new and existing drugs, we need better diagnostics so treatment can start early and disrupt transmission, and new ways to improve the immune response to tuberculosis, either through new vaccines or adjuncts to antimicrobial therapy that modulate the immune-response in favor of the host. Only this combination of prevention and treatment of active and latent infection will allow us to reach the Millennium Development Goal of eradication of Tuberculosis by 2050 [3], and contribute to the new post-2015 goals for poverty reduction and sustainable development [4]. Given the bacterium's airborne route of transmission and highly adaptive nature to humans, this chapter reviews cellular and molecular events in the pathogenesis of *M. tuberculosis*, with emphasis on those applicable to the lung.

2.2 Lung

The lung is responsible for mediating gas exchange across a respiratory surface of 130 m^2, which is more than 60 times the surface area of the body [5–7]. On average, this involves inhalation of 14,000 L of air each day [8] and processing not only of CO_2 and O_2, but also of pollutants, allergens, and microbes from the inhaled air. Efficient gas exchange requires a thin barrier between the air and blood; in some instances this barrier is composed of only one endothelial cell and one epithelial cell, and is less than 2 μm thick. The lung must maintain this thin barrier while clearing inhaled insults [5–7].

Upon inhalation, air passes through the nasal cavity to the pharynx, then to the trachea (Figure 2.1). The trachea contains pseudo-stratified columnar epithelium, goblet cells that secrete mucus, ciliated cells, and short basal stems [49]. Following the trachea are the



Immune Cell	Function during *M. tuberculosis* infection	References
Macrophage	Major niche for *M. tuberculosis;* macrophage susceptibility to *M. tuberculosis* infection affected by tissue site, which phagocytic receptor is engaged, phagosome maturation, autophagy, NLRs, PPARγ activation, miRNAs, and mode of macrophage cell death.	**[9]–[15]**
Neutrophil	Role still being defined. Early in infection: controls *M. tuberculosis* growth, regulates IFNγ expression. Late in infection: enhances *M. tuberculosis* persistence, produces IL-10, causes tissue damage.	**[16]–[20]**
DC	Can harbor *M. tuberculosis* which traffic to phagolysosomes, present antigens. Differentiation state modulated by *M. tuberculosis*. Migrate to lymph nodes following phagocytosis of *M. tuberculosis*. Engulf apoptotic vesicles derived from *M. tuberculosis*-infected macrophages. Prime CD4 T cells, activate CD8 T cells through the classical and "detour" pathways.	**[21]–[28]**
NK Cell	Mediate killing of intracellular *M. tuberculosis*.Directly interact with *M. tuberculosis*, via nanotube-like structures, to augment killing. Interact with DCs and γδT cells.	**[29]–[33]**
B Cell	Mediate humoral responses to *M. tuberculosis*. Generate LAM-specific IgA responses following BCG vaccination. Present in outer lymphocyte aggregations of granulomas. Release MHC-II enriched exosomes that elicit T cell responses.	**[34]–[37]**
T Cell	CD4 and CD8: major producers of IFNγ, activate *M. tuberculosis*-infected APCs to control growth. MAIT: produce IFNγ, lyse infected cells. CD1: recognize *M. tuberculosis* lipid antigens. γδ: release perforin and granulysin to kill intracellular bacteria. Tregs: delay priming of protective T cell responses.	**[38]–[48]**

Figure 2.1 *Schematic of the lung and the role of pulmonary immune cells during M. tuberculosis infection. Top panel: Branching of the airways, culminating in the alveolar sacs and the alveolus. Also depicted are the cells in the alveolus. Bottom Table: A few key roles of immune cells during M. tuberculosis infection are listed. For more details and references, see text. Abbreviations: AEC I and II: Type I and II Alveolar Epithelial Cell, AM: Alveolar Macrophage, APC: Antigen-Presenting Cell, BCG: Mycobacterium bovis bacillus Calmette–Guerin, DC: Dendritic Cell, IM: Interstitial Macrophage, IFNγ: Interferon Gamma, IVM: Intravascular Macrophage, MAIT: Mucosal Associated Invariant T, miRNAs: microRNAs, NK: Natural Killer, PPARγ: Peroxisome Proliferator-Activated Receptor Gamma*

bronchi; the right bronchus is slightly wider and more vertical than the left, which is why inhaled objects predominantly transit the right bronchus [50]. The bronchi split into bronchioles, followed by terminal bronchioles, transitional bronchioles, and respiratory bronchioles. Transitional bronchioles are where the alveoli start to form and serve as the transition between conducting and acinar airways [7]. Respiratory bronchioles split into alveolar ducts and terminate at alveolar sacs. One acinus contains about 10,000 alveoli and there are a total of 480 million alveoli in the adult human lung (Figure 2.1) [5, 6, 51].

The lung uses multiple defenses to clear contaminants from the air, discussed in more detail below. Briefly, basic instincts like the coughing and sneezing reflex serve to remove particulates from the lung. Mucus, together with cilia, forms the mucociliary escalator which transfers particles up the trachea for removal. Also, epithelial cells in the airway secrete antimicrobials including lysozyme and antimicrobial peptides like defensins and cathelicidins [49, 52]. These defenses serve to clear particulates $\geq 5\,\mu m$ in size. Anything smaller passes through the conducting system with velocity, eventually settling in the alveoli, where alveolar cells become responsible for their clearance.

2.2.1 Alveoli

The main three cell types in the alveolus are type I and type II alveolar epithelial cells (AECs) and alveolar macrophages (AMs) (Figure 2.1). These cells are coated by alveolar fluid and surfactant. External to the alveolus are the alveolar septa, which contain blood vessels, fibroblasts, protein fibers, and pores of Kohn. The fibers are responsible for the structural integrity of the alveolus. The pores of Kohn are holes in the septa that connect alveoli to each other, are filled with surfactant, and provide a passage for cells to migrate between alveoli [5, 53].

2.2.1.1 Alveolar Epithelial Cells (AECs)

Although Type I AECs constitute only 8% of peripheral lung cells (a third of the cells in the alveolus), they cover ~95% of the alveolar surface. Their thin flat shape, as well as their contact with endothelial cells of the pulmonary capillaries, provides the necessary thin surface for gas exchange to occur [5, 6]. The cuboidal Type II AECs constitute about 15% of peripheral lung cells, cover 5% of the alveolar surface and have a smaller surface area than Type I AECs, $250\,\mu m^2$ compared with $5{,}000\,\mu m^2$ [54, 55]. Type II AECs contain distinctive lamellar bodies and have apical microvilli. These cells secrete surfactant phospholipids and proteins, as well as lysozyme and antimicrobial peptides in lamellar bodies [52, 54]. Following lung damage, Type II AECs can serve as precursors to Type I cells and self-renew [5, 6, 52]. These cells also express human leukocyte antigen (HLA) class I and II molecules [54], and murine cells can present mycobacterial antigens [56].

2.2.1.2 Alveolar Macrophages (AMs)

AMs constitute the majority of cells collected from bronchoalveolar lavage (BAL) (≥90%). They maintain the alveolar microenvironment, removing debris, dead cells, and microbes. They are long lived, with a half-life of 1–8 months [57, 58], about 40% of total AMs turnover each year [59]. AMs are thought to originate from peripheral blood monocytes following migration into the lung [60], but may also originate from lung macrophages in response to inflammation [57, 61–65].

AMs must exert tightly regulated pro- and anti-inflammatory actions to control infection without damaging the fragile alveolar environment [59, 66]. Thus they exhibit characteristics of M1 (classically activated) and M2 (alternatively activated) macrophages [9]. They express high levels of mannose receptor (MR), scavenger receptor-A (SR-A), toll-like receptor 9 (TLR9), and the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ), and low levels of TLR2 and the co-stimulatory molecules CD80 and CD86 [10, 59, 66]. Expression of PPARγ may be important for differentiation of AMs [67], which are highly phagocytic [68], but have a limited oxidative burst relative to neutrophils or peripheral blood mononuclear cells (PBMCs) [68, 69], and are weakly bactericidal [57]. They are poor antigen presenters [70] and down-regulate the dendritic cells' (DCs) ability to present antigen [71], and suppress lymphocyte activation [72].

2.2.2 The Different Lung Macrophages

The lung contains three types of macrophages, named based on their location: AMs, intravascular macrophages (IVMs), and interstitial macrophages (IMs). IVMs are located in the capillaries on endothelial cells and IMs are in the interstitial space between alveoli [73, 74]. The IVMs and IMs are less understood than are AMs, likely due to the difficulty in isolating them. AMs are readily isolated from BAL following only a few washes, while IMs are obtained in the BAL following many washes [74], and many animals like mice and humans (in contrast to pigs and horses, for example) may not constitutively produce IVMs [73]. In rhesus macaques IMs were shown to have a higher turnover rate and to be shorter lived than AMs [75]. IMs are thought to regulate tissue fibrosis, inflammation, and antigen presentation [76] and to be more pro-inflammatory than AMs [75]. IVMs are phagocytic and may clear erythrocytes and fibrin from the blood [73].

2.2.3 Other Immune Cells in the Lung

A few DCs are located in the alveoli interstitial space, but most are in the conducting airways [5, 77]. In the alveoli, they sit below the AECs and extend membrane protrusions to sample the inner surface of the airway lumen [78]. Following antigen processing and presentation, DCs migrate to local lymph nodes [79] and inducible bronchus-associated lymphoid tissue (BALT) that forms in response to infection or inflammation [80, 81]. There are few lymphatic vessels around the alveoli so alveolar DCs must migrate through the interstitium to access sites of lymphatic drainage [5]. Other immune cells, including T and B cells, are found in low amounts in the interstitium and are discussed below in relation to *M. tuberculosis* infection.

2.3 General Aspects of Mucus and Surfactant

Mucins are the main glycoproteins in mucus and are either tethered to epithelial cells or secreted. They are produced by submucosal glands and goblet cells, club cells, and alveolar cells in the conducting and peripheral airways. They bind particles and microbes to prevent their adherence to host cells and so mediate clearance via the mucociliary escalator [52].

Pulmonary surfactant is produced by Type II AECs in the alveoli and is a complex mixture of lipids and proteins that bathe cells in the alveolus and reduce surface tension to prevent alveolar collapse during expiration. Dipalmitoylphosphatidylcholine (DPPC) is the most abundant phospholipid in surfactant [82], but surfactant also contains surfactant proteins (SPs) SP-A, SP-B, SP-C, and SP-D [83, 84]. In general, SP-B and SP-C maintain stability of the surfactant lipids while SP-A and SP-D are immunomodulators [52, 85]. SP-A enhances apoptotic cell clearance by macrophages and regulates MR activity, the oxidative burst, and negative regulators of inflammation [86–91]; SP-A also enhances macrophage phagocytosis of *M. tuberculosis* [92–95]. In contrast, SP-D agglutinates *M. tuberculosis*, which decreases macrophage phagocytosis. Intriguingly, the bacteria that are still phagocytosed show enhanced phagosome lysosome (PL) fusion and killing [96–98].

2.4 General *M. tuberculosis*

M. tuberculosis divides only once every 20 hours, which may be in part due to its complex cell envelope consisting of peptidoglycan, proteins (although these have been somewhat neglected [99]), and a diverse range of unusual long-chain lipids (e.g., mycolic acids), carbohydrates and combinations thereof [e.g., lipoarabinomannan (LAM), lipomannan (LM), and phosphatidyl-*myo*-inositol mannosides (PIMs)]. Many of these are important in the interaction with the host, with 166 macrophage proteins of diverse function differentially expressed when exposed to mycobacterial cell wall lipids [100]. Several other recent reviews cover the specifics of the structure and biosynthesis of this large group of molecules [101, 102], so this section will concentrate on some examples where recent progress has been made in understanding the biology of the cell wall lipids phthiocerol dimycocerosate (PDIM) and phenolic glycolipid (PGL).

PDIMs are important for *in vivo* infection of mice, as demonstrated by the severe attenuation of mutants deficient in PDIM synthesis or translocation [103–105]. It was subsequently shown that PDIMs are also spontaneously lost *in vitro* [106], impacting the immune response and making it difficult to interpret studies where PDIM status has not been established. How PDIMs facilitate mycobacterial infection *in vivo* has also now been studied; they are important for resisting interferon-γ (IFNγ)-independent immune responses [107], which are important during the early phase, at least of mouse infection, making *M. tuberculosis* strains that do not express PDIMs more susceptible to killing [108]. It has been proposed that PDIMs mask pathogen-associated molecular patterns (PAMPs), and dampen TLR-signaling and the recruitment of macrophages that produce microbicidal reactive nitrogen species [109]. PGLs are also involved in this, but they are not essential for virulence and are sometimes missing from clinical isolates. Loss of PGL correlates with a more inflammatory macrophage phenotype, while overproduction of PGL inhibits the release of pro-inflammatory cytokines [110]. This suggests that these two mycobacterial lipids work in tandem to modulate the host immune response in favor of the pathogen. In the related *M. marinum* zebrafish system, PGL uses a C-C chemokine receptor 2 (CCR2)-mediated pathway to recruit mycobacteria-permissive macrophages that do not produce reactive nitrogen species [109]. Whether this translates to human TB is as yet unclear.

2.5 *M. tuberculosis* Interaction with the Lung Macrophage

2.5.1 Initial Interactions Following Inhalation

Human alveolar lining fluid (ALF) contains hydrolases that alter the *M. tuberculosis* cell wall, reducing exposure of mannose-capped LAM (ManLAM) and trehalose 6,6'-dimycolate (TDM; cord factor). ALF treatment of *M. tuberculosis* reduced its association with and intracellular replication in human macrophages and led to increased tumor necrosis factor (TNF)α release by macrophages [111]. Thus, initial exposure to surfactant may alter the *M. tuberculosis* cell wall before interaction with AMs or AECs and affect subsequent interactions with the host, perhaps by altering the receptor primarily engaged by the bacteria.

2.5.2 Interactions with the Macrophage

2.5.2.1 Phagocytic Receptors

The MR (CD206) is highly expressed by AMs and subsets of DCs, but not by monocytes [112–116]. It is the dominant C-type lectin on human AMs and monocyte-derived macrophages (MDMs) and recognizes endogenous N-linked glycoproteins [117, 118] and mannose-containing PAMPs [119] via its carbohydrate-recognition domains (CRDs). The MR discriminates between mannose-containing PAMPs based on the degree and nature of mannan motifs. It binds to the mannose caps of ManLAM [120, 121] and the higher order PIMs that are more mannosylated and found in greater amounts on pathogenic mycobacteria [11], thus differentiating among *M. tuberculosis* strains [122, 123]. *M. tuberculosis* is thought to use molecular mimicry to bind the MR and mediate favorable entry into macrophages, and usage of the MR may be a marker of host adaptation [121]. Binding to the MR mediates phagocytosis of *M. tuberculosis* and leads to decreased PL fusion [11, 12, 124], acidification [125, 126], and oxidative burst [127], as well as release of anti-inflammatory cytokines [128]. MR engagement also leads to increased PPARγ activity, which allows for enhanced survival of *M. tuberculosis* in the macrophage, discussed in more detail below [10]. The MR can facilitate presentation of lipids and ManLAM and serves as a prototypic pattern-recognition receptor (PRR) linking innate and adaptive immunity, which has been exploited to deliver DNA vaccines to antigen-presenting cells (APCs) [9]; and is being used to modulate the immune system for therapeutic and vaccine purposes [129]. The MR may also contribute to chronic stages of *M. tuberculosis* infection by mediating homotypic cellular adhesion and giant-cell formation [130], which are characteristic of TB granulomas [131, 132].

DC-specific intercellular adhesion molecule 3 grabbing non-integrin (DC-SIGN) is expressed by DCs and subsets of macrophages [115, 133]. Its expression by human macrophages is generally low, but can be induced following *M. tuberculosis* infection [134] or other stimulation [135]. DC-SIGN recognizes mannosylated glycoconjugates like *M. tuberculosis* ManLAM and PIMs [11, 21, 136]. DC-SIGN activation during *M. tuberculosis* infection leads to PL fusion (contrary to the MR) and impedes DC maturation [21, 137].

Macrophage-inducible C-type lectin (Mincle; Clec4e, Clecsf9) is expressed on leukocytes at low levels before activation, but is highly expressed on mouse macrophages following stimulation [138]. Mincle recognizes damaged cells and fungi [139, 140]. It also

recognizes *M. tuberculosis* TDM, resulting in enhanced inflammatory cytokine production and granuloma formation [141, 142] but is not required for control of *M. tuberculosis* infection in mice [143].

Dectin-1 is a non-classical C-type lectin that recognizes β-glucans [144]. It is highly expressed on DCs, macrophages, monocytes, neutrophils, and a subset of T cells [145]. Dectin-1 activation in conjunction with TLR4 during *M. tuberculosis* infection induces an interleukin (IL)-17A response [146]. Similar to MR, Dectin-1 differentiates between mycobacterium strains; activation by non-pathogenic (*M. smegmatis*, *M. phlei*) and attenuated [*M. bovis* bacillus Calmette–Guerin (BCG), *M. tuberculosis* H37Ra] mycobacteria, but not virulent *M. tuberculosis* H37Rv enhances TNFα, IL-6, and regulated on activation, normal T cell expressed and secreted (RANTES) production by macrophages [147, 148]. Dectin-1 activation inhibits replication of BCG, but not virulent *M. tuberculosis*, in human macrophages [149]. Dectin-2 recognizes mannose-containing lipids and is expressed by DC subsets and macrophages [150]. Its stimulation by ManLAM induces pro- and anti-inflammatory responses and promotes T cell-mediated adaptive immunity in mice [151]. Dectin-3 (also called MCL and Clec4d) recognizes TDM, and is required for TDM-induced Mincle expression and production [152, 153].

Complement receptors CR1, CR3, and CR4 are major phagocytic receptors. They are expressed by monocytes, macrophages, neutrophils, and some lymphocytes, and their expression and activities change in a tissue- and differentiation-specific manner. For example, CR4 expression increases during differentiation of monocytes into macrophages and is the prominent CR on AMs [154, 155]. CRs can mediate phagocytosis of opsonized and non-opsonized *M. tuberculosis* by human macrophages [13, 156–158]. CRs recognize surface polysaccharides, PIMs, and glycopeptidolipids of non-opsonized *M. tuberculosis* [159, 160].

FcγRs play a role in the phagocytosis of *M. tuberculosis* following opsonization of bacteria with immune-specific antibody [13]; this leads to enhanced PL fusion [161].

2.5.2.2 Toll-Like Receptors

TLRs are a highly conserved family of transmembrane PRRs that are expressed by many cells, including AMs and DCs [162–166]. TLRs are surface exposed (e.g., TLR2 and TLR4) and intracellular (e.g., TLR9) [163]. They are classically thought of as pro-inflammatory through NFκB activation [167–169], but can also act through the negative regulators TRIF, IRF, and IRAK-M [170, 171]. Recognition of *M. tuberculosis* occurs via TLRs 2, 4 and 9. Mycobacterial lipids [phospho-*myo*-inositol-capped LAM (PILAM), PIM_2 and PIM_6], and 19 kDa lipoprotein are agonists of TLR2 [172, 173]. TLR4 recognizes the heat-shock protein 65 (hsp65) [363] and CpG motifs on the *M. tuberculosis* genome are ligands for TLR9 [174]. Studies in TLR knockout (KO) mice with *M. tuberculosis* have yielded contradictory results. Single and double KO mice exhibit a range of phenotypes in response to *M. tuberculosis* infection, ranging from enhanced mortality and defective IL-12p40 and IFNγ responses [175–178]. Conversely, triple KO TLR2/4/9 mice exhibited no loss of protective T cell responses, and growth of *M. tuberculosis* was similar in wild-type and KO mice [179]. Myeloid differentiation factor 88 (MyD88), an adaptor utilized by most TLRs, was reported to be indispensable for control of mycobacterial growth [179, 180].

2.5.2.3 Scavenger Receptors

There are at least eight different classes of SRs, which cooperate with other receptors to mediate their function [181]. AMs express at least four different SRs, the Class A SR-A isoforms I and II (SRA-I/II) and macrophage receptor with collagenous structure (MARCO) and the Class B receptor CD36. SRA-I/II bind to most polyanionic molecules [182, 183], MARCO removes unopsonized particles in the lung [184], and CD36 removes apoptotic cells and oxidized LDL [185]. SRs mediate *M. tuberculosis* binding to macrophages [186]. MARCO cooperates with TLR2 and CD14 to initiate cytokine release following recognition of *M. tuberculosis* TDM [187]. CD36 contributes to foam cell generation during *M. tuberculosis* infection [188] and PPARγ production during BCG infection [189]. PPARγ induces CD36 expression in human AMs [190]. The specific role of different SRs during *M. tuberculosis* infection is unclear, likely due to a redundancy in scavenger receptor expression [191]. Infection of SRA-I/II [192, 193] and MARCO [194] single KO mice indicates that these SRs may play a role in limiting inflammation and resistance to pulmonary pathogens. CD36 KO mice are more resistant to *M. tuberculosis* infection [195]. Further work is needed to understand the role of SRs during *M. tuberculosis* infection.

2.5.2.4 Phagosome Maturation

Typical phagosome maturation involves sequential fusion of the phagosome with early endosomes, late endosomes, and lysosomes during which process the pH decreases to 4.5–5.0 through the actions of a vacuolar ATPase. The phagosome also acquires antimicrobial peptides and proteases, including cathepsins that are activated by the low pH in the phagosome. These factors all contribute to the bactericidal nature of the mature phagosome and mediate clearance of the ingested particle [196]. However, some pathogens, including *M. tuberculosis*, modify the phagosome such that it becomes a niche for intracellular replication. *M. tuberculosis* phagosomes do not fully acidify, reaching a pH of 6.2, and do not fuse with lysosomes (Rab5, but not Rab7, is acquired). Many *M. tuberculosis* components interfere with this maturation, including ESAT-6/CFP-10 (early secretory antigenic target 6/culture filtrate protein 10), SecA1/2, ManLAM, and TDM [137, 197–200]. ManLAM inhibits PL fusion through the macrophage MR [12]. Recent work showed that a mycobacterial lipoprotein LprG binds ManLAM and controls its distribution in the mycobacterial envelope. Mutants of *M. tuberculosis* lacking LprG have less ManLAM on their surface and are less able to inhibit PL fusion [201, 202].

It has been proposed that *M. tuberculosis* can escape the phagosome and reside in the cytosol. This is controversial, with debate as to whether *M. tuberculosis* replicates in the cytosol or is released in conjunction with macrophage cell death [203]. Cytosolic localization has been proposed because experiments in the 1980–1990s provided electron microscopy images showing intracellular *M. tuberculosis* independent of phagosome membranes [204–206], and components of *M. tuberculosis* were detected in the cytosol following infection in a region of difference-1 (RD-1) and ESAT-6-dependent manner [207–209]. An explanation for the latter observation besides complete phagosome membrane dissolution is that the RD-1-dependent ESX-1 secretion system perforates, but does not destroy, the phagosome membrane, resulting in a ‘leaky’ phagosome [210, 211]. This ‘leaky’ phagosome would allow *M. tuberculosis* components access to the cytosol, and explain how *M. tuberculosis* infection leads to activation of cytosolic immune receptors.

2.5.2.5 Autophagy

Macroautophagy is a major form of autophagy, hereafter referred to simply as autophagy, that involves the entrapment of cytosolic compounds into double-membrane vesicles that fuse with lysosomes to mediate degradation. This process is involved in cell maintenance, and can also be used to limit infection [212–214]. Starvation, rapamycin, TLRs, 2'-5' cyclic GMP-AMP, IL-1, and IFNγ can all induce autophagy [14]. Recent publications have indicated that the host AMP-activated protein kinase-PPARγ, coactivator 1α pathway (AMPK-PPARGC1A) and membrane occupation and recognition nexus repeat containing 2 (MORN2) are involved in autophagy induction and regulate *M. tuberculosis* infection [215, 216]. *M. tuberculosis* has various components that regulate autophagy, including ESAT-6 [211, 217] and the enhanced intracellular survival (*eis*) gene [218]. If autophagy is induced, *M. tuberculosis* colocalizes with the autophagy marker LC3, PL fusion occurs, and *M. tuberculosis* growth is limited [219, 220]. Autophagy is important *in vivo*, autophagy-deficient mice show increased *M. tuberculosis* growth and lung pathology and reduced survival compared with wild-type mice [211, 221]. Two frontline TB drugs, isoniazid and pyrazinamide, induce autophagy in *M. tuberculosis*-infected macrophages and are more inhibitory towards *M. tuberculosis* growth if the autophagy machinery is intact [222]. Manipulation of autophagy is being pursued as a treatment option for TB [223–226].

2.5.2.6 Intracellular Receptors

Intracellular receptors include the transmembrane TLRs and cytosolic nucleotide binding oligomerization domain-containing protein (NOD)-like receptors (NLRs), RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs), and other cytosolic DNA sensors [227–231]. Some of these receptors have been shown to recognize *M. tuberculosis* ligands and play a role during *M. tuberculosis* infection.

NOD1 recognizes D-glutamyl-*meso*-diaminopimelic acid (DAP) while NOD2 recognizes muramyl dipeptide (MDP) and a glycolated form of MDP (GMDP) produced by *M. tuberculosis* [232–234]. NOD2 regulates *M. tuberculosis* growth in human and murine macrophages [235–237] perhaps via release of antimicrobial peptides and autophagy, since MDP increases LL-37, IRGM, and LC3 expression and *M. tuberculosis* killing in AMs [238]. *M. tuberculosis* infection also activates NLRP3; however, NLRP3 function during infection is unclear since NLRP3-deficient mice show a similar susceptibility to *M. tuberculosis* infection as do wild-type mice. However, mice lacking the adaptor protein PYCARD/ASC, which (like NLRP3) is involved in caspase-1 activation, are more susceptible to *M. tuberculosis* infection [239]. AIM2 and the ALR IFI204 recognize DNA and may play a role during *M. tuberculosis* infection [210, 240]; AIM2-deficient mice have a higher bacterial burden and succumb to infection quicker than do wild-type mice [241].

2.5.2.7 PPARγ

The PPARs are a family of nuclear receptor-associated transcription factors. They include PPARα, PPARβ/δ, and PPARγ [242, 243]. PPARγ is highly expressed by AMs, and its deletion leads to increased expression of IFNγ, IL-12, macrophage inflammatory protein 1 alpha (MIP-1α), and inducible nitric oxide synthase (iNOS) [244]. PPARγ expression is induced in macrophages through the MR and TLR2 by *M. tuberculosis* and BCG, but not the avirulent *M. smegmatis* [10, 245]. PPARγ inhibition or knockdown leads to reduced

M. tuberculosis intracellular replication and lipid body formation, and enhanced TNFα production [10, 188, 245]. PPARγ is actively being pursued as a drug target and efforts are ongoing to increase understanding regarding its activities [243].

2.5.2.8 microRNAs (miRNAs)

miRNAs are endogenous, non-coding small RNAs that are typically transcribed from intergenic or intragenic regions of the genome in the pri-miRNA form. Following processing into miRNAs, they bind target mRNAs and typically mediate translational repression or mRNA degradation [246–248]. Recent attention has focused on the specific regulation and function of miRNAs in the lung, particularly regarding cancer and inflammatory responses [249, 250]. miRNAs serve several potential functions during *M. tuberculosis* infection; regulating TLR signaling, NFκB activation, cytokine release, autophagy, and apoptosis to alter *M. tuberculosis* infection and host survival [9]. For example, miR-124 down-regulates expression of MyD88, TRAF6, and TLR6 [251], and miR-let-7f targets a negative regulator of NFκB, A20, to increase cytokine and nitrite production, and reduce *M. tuberculosis* infection [252]. miR-132 and miR-26a negatively regulate the transcriptional coactivator p300 and IFNγ signaling [253]. Expression of miRNAs can be altered in *M. tuberculosis*-infected patients or cells [253–259], sometimes in a virulence-dependent manner, e.g. *M. tuberculosis*, but not *M. smegmatis* infection induces expression of miR-125b [260]. miRNA activity can also be cell-type specific, as miR-19a-3p may regulate expression of 5-lipoxygenase in primary human T cells, but not in B cells [261]. Targeting of miRNAs is a promising host-directed therapy [262].

2.5.2.9 Macrophage Cell Death

Cell death can be an important step in control of infection and, as such, many pathogens manipulate host cell death pathways to enhance their survival [263]. The two cell death pathways that have been most studied are apoptosis, which is commonly thought of as anti-inflammatory and is characterized by retention of cell membrane integrity, and necrosis, which is typically pro-inflammatory and characterized by loss of membrane integrity [264, 265]. Pyroptosis has characteristics of necrosis and apoptosis; pyroptotic cells lose membrane integrity similarly to necrotic cells, but cell death is caspase dependent, similarly to apoptosis [266]. Virulent *M. tuberculosis* inhibits apoptosis and instead induces necrosis to exacerbate infection. Apoptosis prevents *M. tuberculosis* dissemination and enhances antigen presentation to DCs and T cell priming. Necrosis and necroptosis mediate *M. tuberculosis* exit and dissemination from the infected cell, propagating infection; *M. tuberculosis* may down-regulate pyroptosis, but this is not clear [15, 267, 268]. *M. tuberculosis* components involved in regulating cell death include SodA, NuoG, and ESX-1 and ESX-5 [269].

2.6 *M. tuberculosis* Interaction with other Respiratory Immune Cells

2.6.1 Neutrophils

Neutrophils constitute 60% of all leukocytes in the peripheral blood of the host; they have a short life span [270] and are among the first leukocytes to respond to infection. However, the role of neutrophils in defense against *M. tuberculosis* is not clear and the literature is conflicting [271]. Neutrophils are abundantly present in both the sputum and BAL fluid of

patients with active pulmonary TB [272], but analysis of individuals who were in close contact with active TB patients established an inverse correlation between neutrophil count and risk of *M. tuberculosis* infection [273]. Blood transcriptome analysis from TB patients identified a neutrophil-driven IFN-inducible transcript pattern [274], and collectively these studies suggest a direct role for neutrophils in TB pathogenesis. Recruitment of neutrophils to sites of infection is a rapid process, with direct recognition and engulfment of mycobacteria by neutrophils occurring at the site of infection aided by TLR2 [271, 275, 276]. Neutrophils can additionally ingest opsonized mycobacteria and undergo phenotypic changes including increased production of reactive oxygen species (ROS), acquisition of migratory capacity to secondary lymphoid organs [277], and expression of cytokines and chemokines [16, 277]. The ability of neutrophils to kill mycobacteria remains unsettled [279], and further controversy surrounds the ability of human neutrophil peptides to kill mycobacteria [270, 273, 275]. *M. tuberculosis* is reported to survive the oxidative burst in neutrophils and to commit these cells to necrotic death via an RD1-dependent mechanism [280]. Survival of mycobacteria within neutrophils [280–282] leads to bacterial growth, tissue destruction, and bacterial dissemination. CFP-10, an RD1 gene-complex product, is recognized by neutrophils using a chemo-attractant G-protein-coupled receptor, implying a possible role for CFP-10 in the regulation of cell death in neutrophils [283]. Neutrophils are understood to play divergent roles in TB disease [284]; during the initial stages of infection before the arrival of macrophages, neutrophils contribute to a more protective role in the host response by aiding in control of mycobacterial growth [17, 18] and regulating the expression of IFNγ [16]. During the later stage of disease, neutrophils produce IL-10 and aid in the persistence of *M. tuberculosis* [19], transport mycobacteria to draining lymph nodes [277], and cause tissue damage mediated by the presence of excessive IL-17 [20] and impaired IFNγ responses [285]. Neutrophils play a role in the efferocytosis of macrophages infected with mycobacteria at the site of granuloma formation, killing the mycobacteria with ROS [286].

Recent literature suggests that the involvement of neutrophils in the immune response to *M. tuberculosis* has been underestimated; however, the evidence to date remains conflicting [271]. Further work on the defined role of neutrophils in the host defense might also reveal opportunities for new therapeutic interventions.

2.6.2 Dendritic Cells

DCs are a unique subset of immune cells which under steady-state conditions function as sentinels of the immune system. Immature DCs phagocytose *M. tuberculosis* at the site of infection, mature, migrate to secondary lymphoid organs, and prime T cells. DCs are equipped with a repertoire of pathogen-associated and danger-associated molecular pattern receptors. Engagement of individual receptors can ultimately dictate downstream DC responses. TLRs 2, 4, and 9, and DC-SIGN recognize *M. tuberculosis* surface molecules, as discussed above. The interaction between DC-SIGN and ManLAM expressed on virulent mycobacteria is exploited by *M. tuberculosis* to its benefit, with *M. tuberculosis* using DC-SIGN as a portal into DCs; once engulfed, bacteria are targeted to late endosomes/lysosomes expressing LAMP [21]. DC-SIGN-mediated entry leads to IL-10 production and inhibition of DC maturation [21], which in turn causes inefficient T cell priming and a state of antigenic tolerance [22]. Mycobacteria are able to persist in DCs [287, 288]. Other

mycobacterial products like Hip1, a serine hydrolase, modulate DC responses and intracellular survival of the pathogen. Hip1 mutants of *M. tuberculosis* induce high levels of IL-12 and increased expression of the major histocompatibility complex-II (MHC-II) in an MyD88- and TLR2/9-dependent manner [289]. *M. tuberculosis* is also able to retain coronin-1 on the vesicular membrane, which interferes with phagosome maturation and promotes survival of the pathogen [287]. Lung DCs encompass three major subsets of cells: conventional DCs, plasmacytoid DCs, and monocyte-derived DCs, with each subset having specific and interrelating functions in the host [290]. Data from mouse studies show that monocyte-derived DCs are rapid responders and are detected in the lung as early as 48 hours post-infection [291, 292]. DCs undergo phenotypic changes following engulfment of mycobacteria, which include up-regulation of MHC-I/II, CD40, CD80, and CD86 [293], increased production of IL-12, TNFα, IL-1, and IL-6 [294–296], and increased migration to lymph nodes for T cell antigen presentation [23]. In specialized microenvironments, migratory DCs are able to efficiently prime CD4 T cells [24], and activate CD8 T cells by the endosome-cytosol [25] or "detour" [26] antigen-presentation pathway. DCs are a distinctive subset of immune cells that possess a pathway capable of transferring exogenous antigens from the endosome to the cytosol, leading to the presentation of antigens via the classical MHC-I pathway [25, 208]. As part of the "detour pathway", uninfected DCs engulf apoptotic vesicles released from *M. tuberculosis*-infected macrophages and present antigens from these extracellular vesicles to CD8 T cells [26].

DCs are key initiators of the immune response. An emerging paradigm from these studies is the role of DCs as Trojan horses providing a reservoir for *M. tuberculosis* to survive and escape immune surveillance [27, 28]. Characterizing the role of different DC subsets will enhance our understanding of the immune response to *M. tuberculosis*, aiding in the design of a better vaccine.

2.6.3 NK Cells

Natural killer (NK) cells are in the spotlight as the relatively new player in the host response to *M. tuberculosis* and make up 10–15% of the resident lymphocyte population [297]. NK cells are defined phenotypically by the presence of the surface marker CD56 and the absence of CD3, and can be subdivided into two subsets: the first is characterized by low levels of CD56 ($CD56^{dim}$), is $CD16^{hi}$, and is highly cytotoxic; the second, smaller subset is $CD56^{bright}$, $CD16^{lo}$, and displays increased ability to secrete cytokines [297, 298], and pleural fluid from TB patients is enriched with this subset producing IFNγ [299]. NK cells possess lytic activity and are capable of killing intracellular mycobacteria independently of IFNγ and cytotoxic granules [29]. Interestingly, a role for apoptosis, recognition of vimentin by NKp46 and ULBP1 by NKG2D on monocytes/macrophages infected with mycobacteria by NK cells, has been suggested as a mechanism for the killing of intracellular mycobacteria [300–302]. It is well established that NK cell activation is accessory cell-dependent; accessory cells include monocytes, macrophages and DCs [303]. However, an increasingly accepted novel concept is the ability of NK cells to directly interact with *M. tuberculosis* independently of accessory cells: NK cells in direct contact with BCG were able to secrete increased levels of IFNγ and augment their lytic response [30]. NK cells produce nanotube-like structures, which tether mycobacteria and deliver perforin and granulysin directly to mycobacteria, which aids in killing [31]. NK cells in direct contact

with mycobacteria up-regulate the cytotoxicity receptor NKp44 [304]. TLR2 is also involved in the recognition of mycobacteria by NK cells; blocking TLR2 down-regulated IFNγ and reduced cytotoxic activity of NK cells [305]. More recent studies using a panel of purified *M. tuberculosis* cell-wall components identified mycolyl-arabinogalactan-peptidoglycan, mycolic acids, and arabinogalactan as bacterial ligands which bind NKp44 and peptidoglycan as a ligand for TLR2 [306]. Interestingly, Esin et al. presented a two-step model on the role of NKp44 and TLR2 in *M. tuberculosis* infection, with the first step involving direct recognition of mycobacterial cell-wall components, in particular peptidoglycan by TLR2, which leads to the activation of NK cells and augmented expression of NKp44 on their surface. The second step involves the interaction of NKp44 with additional mycobacterial ligands like mycolic acids, which regulate the activation state of NK cells leading to the induction of a protective immune response. On the other hand, prolonged NK cell activation via NKp44 can also lead to exacerbated immunopathology and possibly favors bacterial dissemination [306]. NK cells are able to interact with other cells (e.g., DCs) and regulate responses to *M. tuberculosis*. DCs transform from an immature to a mature state upon recognition of mycobacteria; this change is accompanied by decreased antigen uptake, increased migratory capacity, and enhanced ability to produce TNFα and IL-12 [32, 307]. The up-regulated pro-inflammatory cytokine profile of mature DCs in turn promotes IFNγ release from NK cells. Furthermore, NK cells can negatively regulate DCs, selectively killing immature DCs [32, 308]; in the secondary lymphoid organs, mature DCs that express sub-optimal levels of MHC-I are targets of selective killing by NK cells [32, 309]. The underlying mechanism of the selective targeted killing is unexplored. NK cells can also interact with γδT cells, regulating the production of cytokines from this T cell subset, a process dependent on cell–cell interaction mediated via ICAM-1 [33]. From the available literature [310], we now have a better understanding of the role of NK cell effector functions and their contribution to the maintenance of the regulatory and effector arms of the immune response.

2.6.4 B Cells

The impact of B cell-mediated immune responses during *M. tuberculosis* infection has been largely overlooked in comparison with T cell immune responses in the field of TB immunology. Serum therapy was relatively successful in patients with localized cases of TB rather than chronic disease, in studies performed during the late 19th and early 20th centuries. Unfortunately, experimental discrepancies (lack of appropriate controls and incomparable serum formulations) led to the modest success of serum therapy being discounted and B cells were sidelined. Furthermore, the intracellular nature of *M. tuberculosis* appeared to discount a role for B cells in protective immunity. In recent years, immunological paradigms are changing and it is recognized that responses to intracellular pathogens like *M. tuberculosis* are more likely to be a co-operative affair between cell-mediated and humoral immune responses [34, 311]. Emerging evidence suggests that the host is able to mount an effective humoral response to mycobacterial antigens. For example, LAM-specific immunoglobulin A (IgA) responses were increased in the serum post-BCG vaccination [35]. In other studies, protective effects of passive inoculation using IgA antibodies against α-crystallin were effective at reducing pulmonary bacterial burden [312, 313]. B cells are prominent in the outer lymphocyte aggregates of human TB granulomas [36].

B cell clusters represent sites of rapid cell expansion, which influence the local lung responses. In the lymphocytic cuff of the granuloma, the close proximity of B cells with T cells represents potential antigen-presentation sites [314, 315]. B cells are capable of releasing exosomes that are enriched with MHC-II molecules capable of inducing MHC-II-restricted T cell responses [37]. In the absence of more efficient APCs like DCs, B cells are also likely to be involved in priming naïve CD4 T cells [316, 317]. Although these studies highlight the antigen-presenting capacity of B cells, evidence for this occurring during the course of TB infection is limited and warrants more work. The role of B cells during *M. tuberculosis* infection has been addressed by murine studies, where B cell-deficient mice exhibit aggravated immunopathology associated with neutrophil recruitment and enhanced IL-17 responses in the lungs [318, 319]. Mice deficient in the inhibitory receptor FcγRIIB exhibited reduced bacterial burden and enhanced IFNγ responses in the lungs post *M. tuberculosis* challenge. In contrast, Fcγ chain KO mice display increased mycobacterial growth, corresponding to augmented neutrophil recruitment, increased IL-10 production, and exacerbated pathology. Thus engagement of Fcγ receptors on B cells can have a significant impact on mycobacterial burden and lung pathology [320]. Acute infection in mice lacking B cells resulted in curtailed immunopathology and delayed bacterial dissemination to the spleen [321]. Mice with an intact B cell repertoire but lacking secreted immunoglobulin are highly susceptible to high-dose aerosol infection with *M. tuberculosis* and display altered macrophage activation and elevated levels of IL-10 in the lungs [322]. There is increasing evidence that B cells can regulate the granulomatous immune response serving to control the immunopathology during infection. TB vaccine development is largely concentrated on enhancing T cell-mediated immunity. Published studies place increased emphasis on the protective nature of B cell- and antibody-dependent responses, which should be considered when designing new vaccines and treatment strategies for TB treatment.

2.6.5 T Cells

Dissemination of *M. tuberculosis* to the draining lymph nodes is necessary for the initiation of T cell responses in the host [323, 324]. T cells represent pivotal players in the adaptive immune response to TB infection. Below is a brief overview of T cell subsets and their role in the immune response to mycobacteria.

2.6.5.1 CD4 T Cells

The importance of CD4 T cells during *M. tuberculosis* infection is exemplified in HIV positive individuals. HIV is a dominant risk factor for TB progression from infection to disease, much of which is attributed to the reduced CD4 T cell counts. CD4 T cells are important in the control of *M. tuberculosis* infection since their cytokine repertoire, e.g., IFNγ and TNFα, enhance production of nitric oxide and PL fusion, resulting in mycobacterial killing. The direct interaction of CD4 T cells with MHC-II on macrophages and DCs is necessary to contain intracellular bacteria [38]. The ability of CD4 T cells to produce IFNγ is a key part of the immune response; genetic mutations in IFNγ confer susceptibility to mycobacterial infections in humans [325]. IFNγ KO mice fail to restrict growth of *M. tuberculosis*, exhibit diminished production of NOS, and eventually succumb to infection [326, 327]. IFNγ enhancement of the anti-mycobacterial action of macrophages depends on T cell:macrophage contact [39]. Conflicting reports indicate that the effector function of

CD4 T cells is independent of IFNγ [328, 329], providing insight into the unreliability of IFNγ as a correlate of protection. CD4 T cells also influence the effectiveness of the CD8 T cell response in mice [39], affecting the cytotoxic capacity of CD8 T cells [40] and their ability to produce IFNγ [41].

2.6.5.2 CD8 T Cells

CD8 and CD4 T cells represent the major subsets producing IFNγ in response to *M. tuberculosis* infection [42]. CD8 T cells are renowned for their cytolytic activity mediated by perforin/granzyme and fas/fasL pathways [330]. The ability of *M. tuberculosis* to interact with the cytosol likely enhances the availability of TB antigens for presentation by MHC-I molecules [208]. Cross-priming by DCs after the uptake of apoptotic vesicles also represents a key mechanism of CD8 T cell priming [26]. β2-microglobulin, transporter associated with antigen processing (TAP), or CD8α KO mice displayed phenotypes ranging from increased mortality, extensive pathology, and impaired lymphocytic infiltration [331, 332]. The absence of the RD-1 region in BCG likely contributes to decreased CD8 T cell responses in response to BCG compared with *M. tuberculosis* [333]. A lymphocytic cuff composed of CD4 and CD8 T cells surrounds the TB granuloma, and Ordway et al. report a role for CD8 T cells in its maintenance [43]. The chemokine, XCL1, produced by activated CD8 T cells leads to a reduction of IFNγ-producing CD4 T cells and possibly induces anergy [43]. The role of CD8 T cells is more apparent during the chronic stages of disease, and design of a vaccine stimulating both CD4 and CD8 T cells will aid in a better protective immune response.

2.6.5.3 Mucosal Associated Invariant T Cells

MHC-related protein 1 (MR1)-restricted mucosal associated invariant T (MAIT) cells are unconventional CD8 T cells [334] and are present in the lung airways. MAIT cells detect APCs infected with fungi and bacteria, including *M. tuberculosis*. Following stimulation, MAIT cells produce IFNγ, TNF, and lyse infected cells [44, 45, 335]. They are important *in vivo* during mycobacteria infection: MR1 KO mice show elevated BCG growth compared with wild-type mice [336]. Individuals with active TB have reduced levels of MAIT cells in the peripheral blood and enhanced levels in the lung compared with healthy individuals [44].

2.6.5.4 CD1 T Cells: Sensors of Lipid Antigens

A small number of studies report the importance of the group 1 CD1-restricted T cells in recognizing lipid antigens of *M. tuberculosis*. Upon recognition of lipid antigens, CD1b-restricted T cells release IFNγ and kill intracellular mycobacteria [46]. Recognition of lipid antigens by CD1-restricted T cells occurs *in vivo* [337]. Asymptomatic individuals who have been in contact with mycobacteria or are BCG vaccinated have increased proliferation capacity and secrete significantly higher IFNγ in comparison with healthy donors [338]. Surprisingly, T cells from patients with active TB did not respond to lipid antigens, but initiation of chemotherapy restored the responsiveness of T cells to lipid antigens [338]. Mycobacteria deploy evasion strategies to interfere with lipid presentation and thus restrict the protective immune response.

2.6.5.5 γδT Cells

γδ T cells contribute to TB immunity by producing IFNγ, IL-2, IL-17, and cyototoxic granules. Vγ9Vδ2, a major subset of γδ T cells in peripheral blood, are involved in the direct killing of intracellular bacteria via the release of perforin and granulysin [47]. Vγ9Vδ2 cells are also capable of driving complete maturation of *M. tuberculosis*-infected DCs, characterized by up-regulation of CD40 and CD80. Furthermore, DCs present *M. tuberculosis* phosphoantigens to γδ T cells that drive a selective proliferative response but these T cells are unable to secrete cytokines and cytotoxic granules. The incomplete differentiation of Vγ9Vδ2 T cells has been linked to the absence of IL-15 [339, 340]. Vγ9Vδ2 T cells from active TB patients also exhibit increased proliferative capacity and diminished antimycobacterial responses [341]. γδ T cells play a key role in cross-linking innate and adaptive immunity. The identity of γδ T cell antigens and their role in the cross talk with DCs and immunoregulation remain to be deciphered.

Other minor T cell subsets like regulatory T cells (Treg) are tasked with reduction of IFNγ, enhanced production of IL-10, and delayed priming of protective T cell responses in the lung [48, 342–344]. Further understanding of the regulation of immunity by Treg will be key in understanding the delicate control that precedes the development of a successful protective immune response.

2.7 TB Granuloma

Granulomas are one histopathologic hallmark of TB and the terms "granulomas or tubercles" were described before the causative agent of TB was identified. The link to tubercles was acknowledged in the naming of *M. tuberculosis* [345]. The TB granuloma has been classically thought of as the host response to contain and limit *M. tuberculosis* infection. However, granulomas also provide a niche that is recalcitrant to anti-TB treatment and allows for bacterial persistence.

The center of the 'classic' granuloma consists of different types of macrophages. These include differentiated foamy macrophages and epithelioid cells, and large multinucleated giant cells (fused macrophages). There are occasional NK cells, DCs, and neutrophils mixed with the macrophages. These cells are surrounded by B and T cells, which make up 1–10% and 15–50% of leukocytes in the murine lung, respectively [346]. TB-infected individuals typically have cavitating, necrotic granulomas. Large cavitating granulomas contain loose cellular accumulation and numerous neutrophils and macrophages, along with the characteristic acellular caseous (milky, thick substance) necrotic material, while non-cavitating closed granulomas contain a central necrotic area that is fully acellular [347, 348]. Granulomas are heterogeneous, dynamic structures that contain motile cells and change in size during infection [349–352].

Animal models to study TB granulomas are diverse and each has it benefits and drawbacks (reviewed in [347]). The most common animal model employed is the mouse, which does not generate necrotic granulomas or granuloma structures similar to those found in humans. However, genetically altered mouse models exist that have granuloma structures that resemble the granulomas in humans more closely; for example, mice that over-express IL-13 [353], or lack IL-10 [354] or the *sst1* locus, i.e., C3HeB/FeJ mice [355]. Guinea pigs, rabbits, and non-human primates also generate necrotic, caseating granulomas [356–358].

In vitro PBMC models are also being developed; these lack ultrastructure provided by the lung but represent more tractable human systems [359, 360]. Recent work with a PBMC granuloma model shows that granuloma formation and response are fundamentally different in PBMCs collected from people with latent TB infection (LTBI) compared with naïve individuals, and that *M. tuberculosis* has a unique transcriptional profile in these LTBI-generated granulomas [359]. Finally, *in silico* models of granuloma formation provide additional insight into the mechanisms at play [361, 362].

2.8 Conclusion

As an airborne pathogen, it is essential that we fully understand the nature of transmissible *M. tuberculosis* and its encounter with constituents of the lung alveoli as well as the impact of these events on subsequent granuloma formation and persistence. Our failure to completely understand these events creates a critical barrier in our attempts to develop new effective treatment and vaccine strategies that target the lung (e.g., drug delivery, optimal vaccine responses). The host response to *M. tuberculosis* occurs within host tissues of the lung and elsewhere. In this regard, the granuloma is a specialized tissue microenvironment, akin to the cancer microenvironment, with specialized cells and soluble factors that greatly influence both host and *M. tuberculosis* biology. Although parts of this chapter summarize findings directly applicable to the lung, much of our knowledge is still extrapolated from experiments using cells, and in some cases surrogate bacteria, in systems not representative of the lung environment. As models of the lung become more refined, we will gain valuable new insight into the mechanisms at play, aiding our attempts to improve treatment delivery and activity at the site of infection in the lung.

References

[1] Comas, I., Coscolla, M., Luo, T. *et al*. (2013) Out-of-Africa migration and Neolithic coexpansion of Mycobacterium tuberculosis with modern humans, *Nat. Genet.*, **45**, 1176–1182.
[2] Bos, K.I., Harkins, K.M., Herbig, A. *et al*. (2014) Pre-Columbian mycobacterial genomes reveal seals as a source of New World human tuberculosis, *Nature*, **514**, 494–497.
[3] Reid, A., Grant, A.D., White, R.G. *et al*. (2015) Accelerating progress towards tuberculosis elimination: the need for combination treatment and prevention, *Int. J. Tuberc. Lung Dis.*, **19**, 5–9.
[4] Dye, C. (2014) After 2015: infectious diseases in a new era of health and development, *Philos. Trans. R. Soc. Lond., B, Biol. Sci.*, **369**, 20130426.
[5] Hasenberg, M., Stegemann-Koniszewski, S. and Gunzer, M. (2013) Cellular immune reactions in the lung, *Immunol. Rev.*, **251**, 189–214.
[6] Weibel, E.R. (2009) What makes a good lung?, *Swiss Med. Wkly*, **139**, 375–386.
[7] Burri, P.H. (2011) Development and Growth of the Human Lung, *Compr. Physiol*., Supplement 10: Handbook of Physiology, The Respiratory System, Circulation, and Nonrespiratory Functions, 1–46, First published in 1985.
[8] Hartung, G.H. Myhre, L.G. and Nunneley, S.A. (1980) Physiological effects of cold air inhalation during exercise, *Aviat. Space Environ. Med.*, **51**, 591–594.
[9] Rajaram, M.V.S., Ni, B., Dodd, C.E. and Schlesinger, L.S. (2014) Macrophage immunoregulatory pathways in tuberculosis, *Semin. Immunol.*, **26**, 1–15.
[10] Rajaram, M.V.S., Brooks, M.N., Morris, J.D. *et al*. (2010) Mycobacterium tuberculosis activates human macrophage peroxisome proliferator-activated receptor gamma linking mannose receptor recognition to regulation of immune responses, *J. Immunol.*, **185**, 929–942.

[11] Torrelles, J.B., Azad, A.K. and Schlesinger, L.S. (2006) Fine Discrimination in the Recognition of Individual Species of Phosphatidyl-myo-Inositol Mannosides from Mycobacterium tuberculosis by C-Type Lectin Pattern Recognition Receptors, *J. Immunol.*, **177**, 1805–1816.
[12] Kang, P.B., Azad, A.K., Torrelles, J.B. *et al*. (2005) The human macrophage mannose receptor directs Mycobacterium tuberculosis lipoarabinomannan-mediated phagosome biogenesis, *J. Exp. Med.*, **202**, 987–999.
[13] Schlesinger, L.S., Bellinger-Kawahara, C.G., Payne, N.R. and Horwitz, M.A. (1990) Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3, *J. Immunol.*, **144**, 2771–2780.
[14] Deretic, V. (2014) Autophagy in tuberculosis, *Cold Spring Harbor Perspect. Med.*, **4**, a018481.
[15] Divangahi, M., Behar, S.M. and Remold, H. (2013) Dying to live: how the death modality of the infected macrophage modulates immunity to tuberculosis, *Adv. Exp. Med. Biol.*, **783**, 103–120.
[16] Pedrosa, J., Saunders, B.M., Appelberg, R. *et al*. (2000) Neutrophils play a protective nonphagocytic role in systemic Mycobacterium tuberculosis infection of mice, *Infect. Immun.*, **68**, 577–583.
[17] Fulton, S.A., Reba, S.M., Martin, T.D. and Boom, W.H. (2002) Neutrophil-Mediated Mycobacteriocidal Immunity in the Lung during Mycobacterium bovis BCG Infection in C57BL/6 Mice, *Infect. Immun.*, **70**, 5322–5327.
[18] Sugawara, I., Udagawa, T. and Yamada, H. (2004) Rat Neutrophils Prevent the Development of Tuberculosis, *Infect. Immun.*, **72**, 1804–1806.
[19] Zhang, X., Majlessi, L., Deriaud, E. *et al*. (2009) Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils, *Immunity*, **31**, 761–771.
[20] Cruz, A., Fraga, A.G., Fountain, J.J. *et al*. (2010) Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with Mycobacterium tuberculosis, *J. Exp. Med.*, **207**, 1609–1616.
[21] Geijtenbeek, T.B.H., van Vliet, S.J., Koppel, E.A. *et al*. (2003) Mycobacteria target DC-SIGN to suppress dendritic cell function, *J. Exp. Med.*, **197**, 7–17.
[22] Jonuleit, H. (2000) Induction of Interleukin 10-producing, Nonproliferating CD4+ T Cells with Regulatory Properties by Repetitive Stimulation with Allogeneic Immature Human Dendritic Cells, *J. Exp. Med.*, **192**, 1213–1222.
[23] Humphreys, I.R., Stewart, G.R., Turner, D.J. *et al*. (2006) A role for dendritic cells in the dissemination of mycobacterial infection, *Microbes Infect.*, **8**, 1339–1346.
[24] Khader, S.A., Partida-Sanchez, S., Bell, G. *et al*. (2006) Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection, *J. Exp. Med.*, **203**, 1805–1815.
[25] Rodriguez, A., Regnault, A., Kleijmeer, M. *et al*. (1999) Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells, *Nat. Cell Biol.*, **1**, 362–368.
[26] Schaible, U.E., Winau, F, Sieling, P.A. *et al*. (2003) Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis, *Nat. Med.*, **9**, 1039–1046.
[27] Kaufmann, S.H.E. and Schaible, U.E. (2002) A Dangerous Liaison between Two Major Killers: Mycobacterium tuberculosis and HIV Target Dendritic Cells through DC-SIGN, *J Exp Med.*, **197**, 1–5 (2002).
[28] Kooyk, Y.V., Appelmelk, B. and Geijtenbeek, T.B.H. (2003) A fatal attraction: Mycobacterium tuberculosis and HIV-1 target DC-SIGN to escape immune surveillance, *Trends Mol. Med.*, **9**, 153–159.
[29] Brill, K.J., Li, Q., Larkin, R. *et al*. (2001) Human Natural Killer Cells Mediate Killing of Intracellular Mycobacterium tuberculosis H37Rv via Granule-Independent Mechanisms, *Infect. Immun.*, **69**, 1755–1765.
[30] Esin, S., Batoni, G., Pardini, M. *et al*. (2004) Functional characterization of human natural killer cells responding to Mycobacterium bovis bacille Calmette-Guerin, *Immunology*, **112**, 143–152.
[31] Lu, C.-C., Wu, T.-S., Hsu, Y.-J. *et al*. (2014) NK cells kill mycobacteria directly by releasing perforin and granulysin, *J. Leukoc. Biol.*, **96**, 1119–1129.
[32] Moretta, A. (2002) Natural killer cells and dendritic cells: rendezvous in abused tissues, *Nat. Rev. Immunol.*, **2**, 957–965.

[33] Zhang, R., Zheng, X., Li, B. *et al*. (2006) Human NK Cells Positively Regulate γδ T Cells in Response to Mycobacterium tuberculosis, *J. Immunol.*, **176**, 2610–2616.
[34] Casadevall, A. (2003) Antibody-mediated immunity against intracellular pathogens: two-dimensional thinking comes full circle, *Infect. Immun.*, **71**, 4225–4228.
[35] Brown, R.M., Cruz, O., Brennan, M. *et al*. (2003) Lipoarabinomannan-reactive human secretory immunoglobulin A responses induced by mucosal bacille Calmette-Guérin vaccination, *J. Infect. Dis.*, **187**, 513–517.
[36] Ulrichs, T., Kosmiadi, G.A., Trusov, V. *et al*. (2004) Human tuberculous granulomas induce peripheral lymphoid follicle-like structures to orchestrate local host defence in the lung, *J. Pathol.*, **204**, 217–228.
[37] Raposo, G. (1996) B lymphocytes secrete antigen-presenting vesicles, *J. Exp. Med.*, **183**, 1161–1172.
[38] Srivastava, S. and Ernst, J.D. (2013) Cutting Edge: Direct Recognition of Infected Cells by CD4 T Cells Is Required for Control of Intracellular Mycobacterium tuberculosis In Vivo, *J. Immunol.*, **191**, 1016–1020.
[39] Green, A.M., DiFazio, R. and Flynn, J.L. (2013) IFN-γ from CD4 T Cells Is Essential for Host Survival and Enhances CD8 T Cell Function during Mycobacterium tuberculosis Infection, *J. Immunol.*, **190**, 270–277.
[40] Serbina, N.V., Lazarevic, V. and Flynn, J.L. (2001) CD4+ T Cells Are Required for the Development of Cytotoxic CD8+ T Cells During Mycobacterium tuberculosis Infection, *J. Immunol.*, **167**, 6991–7000.
[41] Bold, T.D. and Ernst, J.D. (2012) CD4+ T cell-dependent IFN-γ production by CD8+ effector T cells in Mycobacterium tuberculosis infection, *J. Immunol.*, **189**, 2530–2536.
[42] Pathan, A.A., Wilkinson, K.A., Wilkinson, R.J. *et al*. (2000) High frequencies of circulating IFN-γ-secreting CD8 cytotoxic T cells specific for a novel MHC class I-restricted Mycobacterium tuberculosis epitope in M tuberculosis-infected subjects without disease, *Eur. J. Immunol.*, **30**, 2713–2721.
[43] Ordway, D., Higgins, D.M., Sanchez-Campillo, J. *et al*. (2007) XCL1 (lymphotactin) chemokine produced by activated CD8 T cells during the chronic stage of infection with Mycobacterium tuberculosis negatively affects production of IFN-γ by CD4 T cells and participates in granuloma stability, *J. Leukoc. Biol.*, **82**, 1221–1229.
[44] Gold, M.C., Cerri, S., Smyk-Pearson, S. *et al*. (2010) Human mucosal associated invariant T cells detect bacterially infected cells, *PLoS Biol.*, **8**, e1000407.
[45] Le Bourhis, L., Dusseaux, M., Bohineust, A. *et al*. (2013) MAIT cells detect and efficiently lyse bacterially-infected epithelial cells, *PLoS Pathog.*, **9**, e1003681.
[46] Gilleron, M., Stenger, S., Mazorra, Z. *et al*. (2004) Diacylated sulfoglycolipids are novel mycobacterial antigens stimulating CD1-restricted T cells during infection with Mycobacterium tuberculosis, *J. Exp. Med.*, **199**, 649–659.
[47] Dieli, F., Troye-Blomberg, M., Ivanyi, J. *et al*. (2001) Granulysin-Dependent Killing of Intracellular and Extracellular Mycobacterium tuberculosis by Vγ9/Vδ2 T Lymphocytes, *J. Infect. Dis.*, **184**, 1082–1085.
[48] Shafiani, S., Tucker-Heard, G., Kariyone. A. *et al*. (2010) Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis, *J. Exp. Med.*, **207**, 1409–1420.
[49] Ryu, J.-H., Kim, C.-H. and Yoon, J.-H. (2010) Innate immune responses of the airway epithelium, *Mol. Cells*, **30**, 173–183.
[50] Saladin, K.S. (2010) Anatomy and Physiology, Fifth Edition, McGraw-Hill.
[51] Weibel, E.R. (2008) How to make an alveolus, *Eur. Respir. J.*, **31**, 483–485.
[52] Whitsett, J.A. and Alenghat, T. (2015) Respiratory epithelial cells orchestrate pulmonary innate immunity, *Nat. Immunol.*, **16**, 27–35.
[53] Bastacky, J. and Goerke, J. (1992) Pores of Kohn are filled in normal lungs: low-temperature scanning electron microscopy, *J. Appl. Physiol.*, **73**, 88–95.
[54] Mason, R.J. (2006) Biology of alveolar type II cells, *Respirology*, **11 Suppl**, S12–S15.
[55] Guillot, L., Nathan, N., Tabary, O. *et al*. (2013) Alveolar epithelial cells: Master regulators of lung homeostasis, *Int. J. Biochem. Cell. Biol.*, **45**, 2568–2573.

[56] Debbabi, H. (2005) Primary type II alveolar epithelial cells present microbial antigens to antigen-specific CD4+ T cells, *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **289**, L274–L279.
[57] Fels, A.O. and Cohn, Z.A. (1986) The alveolar macrophage, *J. Appl. Physiol.*, **60**, 353–369.
[58] Murphy, J., Summer, R., Wilson, A.A. *et al*. (2008) The Prolonged Life-Span of Alveolar Macrophages, *Am. J. Respir. Cell Mol. Biol.*, **38**, 380–385.
[59] Hussell, T. and Bell, T.J. (2014) Alveolar macrophages: plasticity in a tissue-specific context, *Nat. Rev. Immunol.*, **14**, 81–93.
[60] van Oud Alblas, A.B. and van Furth, R. (1979) Origin, Kinetics, and characteristics of pulmonary macrophages in the normal steady state, *J. Exp. Med.*, **149**, 1504–1518.
[61] Bitterman, P.B., Saltzman, L.E., Adelberg, S. *et al*. (1984) Alveolar macrophage replication One mechanism for the expansion of the mononuclear phagocyte population in the chronically inflamed lung, *J. Clin. Invest.*, **74**, 460–469.
[62] Landsman, L. and Jung, S. (2007) Lung macrophages serve as obligatory intermediate between blood monocytes and alveolar macrophages, *J. Immunol.*, **179**, 3488–3494.
[63] Kopf, M., Schneider, C. and Nobs, S.P. (2014) The development and function of lung-resident macrophages and dendritic cells, *Nat. Immunol.*, **16**, 36–44.
[64] Epelman, S., Lavine, K.J. and Randolph, G.J. (2014) Origin and Functions of Tissue Macrophages, *Immunity*, **41**, 21–35.
[65] Ginhoux, F. (2014) Fate PPAR-titioning: PPAR-γ "instructs" alveolar macrophage development, *Nat. Immunol.*, **15**, 1005–1007.
[66] Lambrecht, B.N. (2006) Alveolar macrophage in the driver's seat, *Immunity*, **24**, 366–368.
[67] Schneider, C., Nobs, S.P., Kurrer, M. *et al*. (2014) Induction of the nuclear receptor PPAR-gamma; by the cytokine GM-CSF is critical for the differentiation of fetal monocytes into alveolar macrophages, *Nat. Immunol.*, **15**, 1–14.
[68] Hoidal, J.R., Schmeling, D. and Peterson, P.K. (1981) Phagocytosis, bacterial killing, and metabolism by purified human lung phagocytes, *J. Infect. Dis.*, **144**, 61–71.
[69] Greening, A.P. and Lowrie, D.B. (1983) Extracellular release of hydrogen peroxide by human alveolar macrophages: the relationship to cigarette smoking and lower respiratory tract infections, *Clin. Sci.*, **65**, 661–664.
[70] Lyons, C.R., Ball, E.J., Toews, G.B. *et al*. (1986) Inability of human alveolar macrophages to stimulate resting T cells correlates with decreased antigen-specific T cell-macrophage binding, *J. Immunol.*, **137**, 1173–1180.
[71] Holt, P.G., Oliver, J., Bilyk, N. *et al*. (1993) Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages, *J. Exp. Med.*, **177**, 397–407.
[72] Roth, M.D. and Golub, S.H. (1993) Human pulmonary macrophages utilize prostaglandins and transforming growth factor beta 1 to suppress lymphocyte activation, *J. Leukoc. Biol.*, **53**, 366–371.
[73] Schneberger, D., Aharonson-Raz, K. and Singh, B. (2012) Pulmonary intravascular macrophages and lung health: What are we missing?, *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **302**, L498–L503.
[74] Lohmann-Matthes, M.-L., Steinmüller, C. and Franke-Ullmann, G. (1994) Pulmonary macrophages, *Eur. Respir. J.*, **7**, 1678–1689.
[75] Cai, Y., Sugimoto, C., Arainga, M. *et al*. (2014) In vivo characterization of alveolar and interstitial lung macrophages in rhesus macaques: implications for understanding lung disease in humans, *J. Immunol.*, **192**, 2821–2829.
[76] Schneberger, D., Aharonson-Raz, K. and Singh, B. (2010) Monocyte and macrophage heterogeneity and Toll-like receptors in the lung, *Cell Tissue Res.*, **343**, 97–106.
[77] Guilliams, M., Lambrecht, B.N. and Hammad, H. (2013) Division of labor between lung dendritic cells and macrophages in the defense against pulmonary infections, *Mucosal Immunol.*, **6**, 464–473.
[78] Thornton, E.E., Looney, M.R., Bose, O. *et al*. (2012) Spatiotemporally separated antigen uptake by alveolar dendritic cells and airway presentation to T cells in the lung, *J. Exp. Med.*, **209**, 1183–1199.
[79] Vermaelen, K.Y., Carro-Muino, I., Lambrecht, B.N. and Pauwels, R.A. (2001) Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes, *J. Exp. Med.*, **193**, 51–60.

[80] Halle, S., Dujardin, H.C., Bakocevic, N. *et al*. (2009) Induced bronchus-associated lymphoid tissue serves as a general priming site for T cells and is maintained by dendritic cells, *J. Exp. Med.*, **206**, 2593–2601.
[81] Randall, T.D. (2010) Pulmonary dendritic cells: thinking globally, acting locally, *J. Exp. Med.*, **207**, 451–454.
[82] Wright, J.R. (1997) Immunomodulatory functions of surfactant, *Physiol. Rev.*, **77**, 931–962.
[83] Wright, J.R. (2005) Immunoregulatory functions of surfactant proteins, *Nat. Rev. Immunol.*, **5**, 58–68.
[84] Mason, R.J. and Voelker, D.R. (1998) Regulatory mechanisms of surfactant secretion, *Biochim. Biophys. Acta*, **1408**, 226–240.
[85] Crouch, E. and Wright, J.R. (2001) Surfactant proteins A and D and pulmonary host defense, *Annu. Rev. Physiol.*, **63**, 521–554.
[86] Beharka, A.A., Gaynor, C.D., Kang, B.K. *et al*. (2002) Pulmonary surfactant protein A up-regulates activity of the mannose receptor, a pattern recognition receptor expressed on human macrophages, *J. Immunol.*, **169**, 3565–3573.
[87] Crowther, J.E., Kutala, V.K., Kuppusamy, P. *et al*. (2004) Pulmonary surfactant protein A inhibits macrophage reactive oxygen intermediate production in response to stimuli by reducing NADPH oxidase activity, *J. Immunol.*, **172**, 6866–6874.
[88] Nguyen, H.A., Rajaram, M.V.S., Meyer, D.A. and Schlesinger, L.S. (2012) Pulmonary surfactant protein A and surfactant lipids upregulate IRAK-M, a negative regulator of TLR-mediated inflammation in human macrophages, *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **303**, L608–L616.
[89] van Iwaarden, F., Welmers, B., Verhoef, J. *et al*. (1990) Pulmonary surfactant protein A enhances the host-defense mechanism of rat alveolar macrophages, *Am. J. Respir. Cell Mol. Biol.*, **2**, 91–98.
[90] Haagsman, H.P. (1998) Interactions of surfactant protein A with pathogens, *Biochim. Biophys. Acta*, **1408**, 264–277.
[91] Carlson, T.K., Brooks, M.N, Rajaram, M.V.S. *et al*. (2010) Pulmonary Innate Immunity: Soluble and Cellular Host Defenses of the Lung, in Regulation of Innate Immune Function (eds Marsh, C.B., Piper, M.G. and Tridandapani, S.), Transworld Research Signpost, STM Books.
[92] Gaynor, C.D., McCormack, F.X., Voelker, D.R. *et al*. (1995) Pulmonary surfactant protein A mediates enhanced phagocytosis of Mycobacterium tuberculosis by a direct interaction with human macrophages, *J. Immunol.*, **155**, 5343–5351.
[93] Downing, J.F., Pasula, R., Wright, J.R. *et al*. (1995) Surfactant protein A promotes attachment of Mycobacterium tuberculosis to alveolar macrophages during infection with human immunodeficiency virus, *Proc. Natl. Acad. Sci. USA*, **92**, 4848–4852.
[94] Pasula, R., Downing, J.F., Wright, J.R. *et al*. (1997) Surfactant protein A (SP-A) mediates attachment of Mycobacterium tuberculosis to murine alveolar macrophages, *Am. J. Respir. Cell Mol. Biol.*, **17**, 209–217.
[95] Sidobre, S., Nigou, J., Puzo, G. and Riviere, M. (2000) Lipoglycans are putative ligands for the human pulmonary surfactant protein A attachment to mycobacteria. Critical role of the lipids for lectin-carbohydrate recognition, *J. Biol. Chem.*, **275**, 2415–2422.
[96] Ferguson, J.S., Voelker, D.R., McCormack, F.X. and Schlesinger, L.S. (1999) Surfactant protein D binds to Mycobacterium tuberculosis bacilli and lipoarabinomannan via carbohydrate-lectin interactions resulting in reduced phagocytosis of the bacteria by macrophages, *J. Immunol.*, **163**, 312–321.
[97] Ferguson, J.S., Martin, J.L., Azad, A.K. *et al*. (2006) Surfactant protein D increases fusion of Mycobacterium tuberculosis-containing phagosomes with lysosomes in human macrophages, *Infect. Immun.*, **74**, 7005–7009.
[98] Ferguson, J.S., Voelker, D.R., Ufnar, J.A. *et al*. (2002) Surfactant protein D inhibition of human macrophage uptake of Mycobacterium tuberculosis is independent of bacterial agglutination, *J. Immunol.*, **168**, 1309–1314.
[99] Niederweis, M., Danilchanka, O., Huff, J. *et al*. (2010) Mycobacterial outer membranes: in search of proteins, *Trends Microbiol.*, **18**, 109–116.

[100] Shui, W., Gilmore, S.A., Sheu, L. *et al*. (2009) Quantitative Proteomic Profiling of Host–Pathogen Interactions: The Macrophage Response to Mycobacterium tuberculosis Lipids, *J. Proteome Res.*, **8**, 282–289.
[101] Marrakchi, H., Lanéelle, M.-A. and Daffe, M. (2014) Mycolic Acids: Structures, Biosynthesis, and Beyond, *Chem. Biol.*, **21**, 67–85.
[102] Mishra, A.K., Driessen, N.N., Appelmelk, B.J. and Besra, G.S. (2011) Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in Mycobacterium tuberculosis physiology and host-pathogen interaction, *FEMS Microbiol. Rev.*, **35**, 1126–1157.
[103] Domenech, P., Reed, M.B. and Barry, C.E. (2005) Contribution of the Mycobacterium tuberculosis MmpL Protein Family to Virulence and Drug Resistance, *Infect. Immun.*, **73**, 3492–3501.
[104] Cox, J.S., Chen, B., McNeil, M. and Jacobs, W.R. (1999) Complex lipid determines tissue-specific replication of Mycobacterium tuberculosis in mice, *Nature*, **402**, 79–83.
[105] Camacho, L.R., Ensergueix, D., Perez, E. *et al*. (1999) Identification of a virulence gene cluster of Mycobacterium tuberculosis by signature-tagged transposon mutagenesis, *Mol. Microbiol.*, **34**, 257–267.
[106] Domenech, P. and Reed, M.B. (2009) Rapid and spontaneous loss of phthiocerol dimycocerosate (PDIM) from Mycobacterium tuberculosis grown in vitro: implications for virulence studies, *Microbiology (Reading, Engl).*, **155**, 3532–3543.
[107] Murry, J.P., Pandey, A.K., Sassetti, C.M. and Rubin, E.J. (2009) Phthiocerol Dimycocerosate Transport Is Required for Resisting Interferon-γ–Independent Immunity, *J. Infect. Dis.*, **200**, 774–782.
[108] Day, T.A., Mittler, J.E., Nixon, M.R. *et al*. (2014) Mycobacterium tuberculosis Strains Lacking Surface Lipid Phthiocerol Dimycocerosate Are Susceptible to Killing by an Early Innate Host Response, *Infect. Immun.*, **82**, 5214–5222.
[109] Cambier, C.J., Takaki, K.K., Larson, R.P. *et al*. (2014) Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids, *Nature*, **505**, 218–222.
[110] Reed, M.B., Domenech, P., Manca, C. *et al*. (2004) A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response, *Nature*, **431**, 84–87.
[111] Arcos, J., Sasindran, S.J., Fujiwara, N. *et al*. (2011) Human lung hydrolases delineate Mycobacterium tuberculosis-macrophage interactions and the capacity to control infection, *J. Immunol.*, **187**, 372–381.
[112] Stahl, P.D. (1990) The macrophage mannose receptor: current status, *Am. J. Respir. Cell Mol. Biol.*, **2**, 317–318.
[113] Speert, D.P. and Silverstein, S.C. (1985) Phagocytosis of unopsonized zymosan by human monocyte-derived macrophages: maturation and inhibition by mannan, *J. Leukoc. Biol.*, **38**, 655–658.
[114] Wileman, T.E., Lennartz, M.R. and Stahl, P.D. (1986) Identification of the macrophage mannose receptor as a 175-kDa membrane protein, *Proc. Natl. Acad. Sci. USA*, **83**, 2501–2505.
[115] McGreal, E.P., Miller, J.L. and Gordon, S. (2005) Ligand recognition by antigen-presenting cell C-type lectin receptors, *Curr. Opin. Immunol.*, **17**, 18–24.
[116] Stahl, P.D. and Ezekowitz, R.A. (1998) The mannose receptor is a pattern recognition receptor involved in host defense, *Curr. Opin. Immunol.*, **10**, 50–55.
[117] Martinez-Pomares, L., Linehan, S.A., Taylor, P.R. and Gordon, S. (2001) Binding properties of the mannose receptor, *Immunobiology*, **204**, 527–535.
[118] Lee, S.J., Evers, S., Roeder, D. *et al*. (2002) Mannose receptor-mediated regulation of serum glycoprotein homeostasis, *Science*, **295**, 1898–1901.
[119] Medzhitov, R. and Janeway, C. (2000) Innate immunity, *N. Engl. J. Med.*, **343**, 338–344.
[120] Schlesinger, L.S., Hull, S.R. and Kaufman, T.M. (1994) Binding of the terminal mannosyl units of lipoarabinomannan from a virulent strain of Mycobacterium tuberculosis to human macrophages, *J. Immunol.*, **152**, 4070–4079.
[121] Torrelles, J.B. and Schlesinger, L.S. (2010) Diversity in Mycobacterium tuberculosis mannosylated cell wall determinants impacts adaptation to the host, *Tuberculosis (Edinburgh)*, **90**, 84–93.

[122] Schlesinger, L.S., Kaufman, T.M., Iyer, S. *et al*. (1996) Differences in mannose receptor-mediated uptake of lipoarabinomannan from virulent and attenuated strains of Mycobacterium tuberculosis by human macrophages, *J. Immunol.*, **157**, 4568–4575.
[123] Torrelles, J.B., Knaup, R., Kolareth, A. *et al*. (2008) Identification of Mycobacterium tuberculosis Clinical Isolates with Altered Phagocytosis by Human Macrophages Due to a Truncated Lipoarabinomannan, *J. Biol. Chem.*, **283**, 31417–31428.
[124] Aderem, A. and Underhill, D. (1999) Mechanisms of phagocytosis in macrophages, *Annu. Rev. Immunol.*, **17**, 593–623.
[125] Singh, C.R., Moulton, R.A., Armitige, L.Y. *et al*. (2006) Processing and presentation of a mycobacterial antigen 85B epitope by murine macrophages is dependent on the phagosomal acquisition of vacuolar proton ATPase and in situ activation of cathepsin D, *J. Immunol.*, **177**, 3250–3259.
[126] Sturgill-Koszycki, S., Schlesinger, P.H., Chakraborty, P. *et al*. (1994) Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase, *Science*, **263**, 678–681.
[127] Astarie-Dequeker, C., N'Diaye, E.N., Le Cabec, V. *et al*. (1999) The mannose receptor mediates uptake of pathogenic and nonpathogenic mycobacteria and bypasses bactericidal responses in human macrophages, *Infect. Immun.*, **67**, 469–477.
[128] Chieppa, M., Bianchi, G., Doni, A. *et al*. (2003) Cross-linking of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program, *J. Immunol.*, **171**, 4552–4560.
[129] Azad, A.K., Rajaram, M.V.S. and Schlesinger, L.S. (2014) Exploitation of the Macrophage Mannose Receptor (CD206) in Infectious Disease Diagnostics and Therapeutics, *J. Cytol. Mol. Biol.*, **1**.
[130] McNally, A.K., DeFife, K.M. and Anderson, J.M. (1996) Interleukin-4-induced macrophage fusion is prevented by inhibitors of mannose receptor activity, *Am. J. Pathol.*, **149**, 975–985.
[131] Gordon, S. and Martinez, F.O. (2010) Alternative Activation of Macrophages: Mechanism and Functions, *Immunity*, **32**, 593–604.
[132] Gordon, S., Keshav, S. and Stein, M. (1994) BCG-induced granuloma formation in murine tissues, *Immunobiology*, **191**, 369–377.
[133] Bleijs, D.A., Geijtenbeek, T.B.H., Figdor, C.G. and van Kooyk, Y. (2001) DC-SIGN and LFA-1: a battle for ligand, *Trends Immunol.*, **22**, 457–463.
[134] Tailleux, L., Pham-Thi, N., Bergeron-Lafaurie, A. *et al*. (2005) DC-SIGN induction in alveolar macrophages defines privileged target host cells for mycobacteria in patients with tuberculosis, *PLoS Med.*, **2**, e381.
[135] Puig-Kröger, A., Serrano-Gomez, D., Caparros, E. *et al*. (2004) Regulated expression of the pathogen receptor dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin in THP-1 human leukemic cells, monocytes, and macrophages, *J. Biol. Chem.*, **279**, 25680–25688.
[136] Tailleux, L., Schwartz, O., Herrmann, J.-L. *et al*. (2002) DC-SIGN Is the Major Mycobacterium tuberculosis Receptor on Human Dendritic Cells, *J. Exp. Med.*, **197**, 121–127.
[137] Guirado, E., Schlesinger, L.S. and Kaplan, G. (2013) Macrophages in tuberculosis: friend or foe, *Semin. Immunopathol.*, **35**, 563–583.
[138] Matsumoto, M., Tanaka, T., Kaisho, T. *et al*. (1999) A novel LPS-inducible C-type lectin is a transcriptional target of NF-IL6 in macrophages, *J. Immunol.*, **163**, 5039–5048.
[139] Yamasaki, S., Matsumoto, M., Takeuchi, O. *et al*. (2009) C-type lectin Mincle is an activating receptor for pathogenic fungus, Malassezia, *Proc. Natl. Acad. Sci. USA*, **106**, 1897–1902.
[140] Yamasaki, S., Ishikawa, E., Sakuma, M. *et al*. (2008) Mincle is an ITAM-coupled activating receptor that senses damaged cells, *Nat. Immunol.*, **9**, 1179–1188.
[141] Ishikawa, E., Ishikawa, T., Morita, Y.S. *et al*. (2009) Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle, *J. Exp. Med.*, **206**, 2879–2888.
[142] Schoenen, H., Bodendorfer, B., Hitchens, K. *et al*. (2010) Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate, *J. Immunol.*, **184**, 2756–2760.

[143] Heitmann, J., Schoenen, H., Ehlers, S. *et al*. (2013) Mincle is not essential for controlling Mycobacterium tuberculosis infection, *Immunobiology*, **218**, 506–516.
[144] Tsoni, S.V. and Brown, G.D. (2008) beta-Glucans and dectin-1, *Ann. N. Y. Acad. Sci.*, **1143**, 45–60.
[145] Taylor, P.R., Brown, G.D., Reid, D.M. *et al*. (2002) The β-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages, *J. Immunol.*, **169**, 3876–3882.
[146] Van De Veerdonk, F.L., Teirlinck, A.C., Kleinnijenhuis, J. *et al*. (2010) Mycobacterium tuberculosis induces IL-17A responses through TLR4 and dectin-1 and is critically dependent on endogenous IL-1, *J. Leukoc. Biol.*, **88**, 227–232.
[147] Rothfuchs, A.G., Bafica, A., Feng, C.G. *et al*. (2007) Dectin-1 interaction with Mycobacterium tuberculosis leads to enhanced IL-12p40 production by splenic dendritic cells, *J. Immunol.*, **179**, 3463–3471.
[148] Yadav, M. and Schorey, J.S (2006) The beta-glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria, *Blood*, **108**, 3168–3175.
[149] Betz, B.E., Azad, A.K., Morris, J.D. *et al*. (2011) β-Glucans inhibit intracellular growth of Mycobacterium bovis BCG but not virulent Mycobacterium tuberculosis in human macrophages, *Microb. Pathog.*, **51**, 233–242.
[150] Taylor, P.R., Reid, D.M., Heinsbroek, S.E.M. *et al*. (2005) Dectin-2 is predominantly myeloid restricted and exhibits unique activation-dependent expression on maturing inflammatory monocytes elicited in vivo, *Eur. J. Immunol.*, **35**, 2163–2174.
[151] Yonekawa, A., Saijo, S., Hoshino, Y. *et al*. (2014) Dectin-2 is a direct receptor for mannose-capped lipoarabinomannan of mycobacteria, *Immunity*, **41**, 402–413.
[152] Zhao, X.-Q., Zhu, L.-L., Chang, Q. *et al*. (2014) C-type lectin receptor dectin-3 mediates trehalose 6,6'-dimycolate (TDM)-induced Mincle expression through CARD9/Bcl10/MALT1-dependent nuclear factor (NF)-kB activation, *J. Biol. Chem.*, **289**, 30052–30062.
[153] Miyake, Y., Toyonaga, K., Mori, D. *et al*. (2013) C-type Lectin MCL Is an FcRgamma-Coupled Receptor that Mediates the Adjuvanticity of Mycobacterial Cord Factor, *Immunity*, **38**, 1050–1062.
[154] Myones, B.L., Dalzell, J.G., Hogg, N. and Ross, G.D. (1988) Neutrophil and monocyte cell surface p150,95 has iC3b-receptor (CR4) activity resembling CR3, *J. Clin. Invest.*, **82**, 640–651.
[155] Arnaout, M.A. (1990) Structure and function of the leukocyte adhesion molecules CD11/CD18, *Blood*, **75**, 1037–1050.
[156] Schlesinger, L.S., Azad, A.K., Torrelles, J.B. *et al*. (2008) Determinants of phagocytosis, phagosome biogenesis and autophagy for Mycobacterium tuberculosis, *in* Handbook of Tuberculosis: Immunology and Cell Biology (eds S.H.E. Kaufmann and W.J. Britton), Wiley VCH Publishers.
[157] Ferguson, J.S., Weis, J.J., Martin, J.L. and Schlesinger, L.S. (2004) Complement protein C3 binding to Mycobacterium tuberculosis is initiated by the classical pathway in human bronchoalveolar lavage fluid, *Infect. Immun.*, **72**, 2564–2573.
[158] Schlesinger, L.S. (1993) Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors, *J. Immunol.*, **150**, 2920–2930.
[159] Cywes, C., Hoppe, H.C., Daffe, M. and Ehlers, M.R. (1997) Nonopsonic binding of Mycobacterium tuberculosis to complement receptor type 3 is mediated by capsular polysaccharides and is strain dependent, *Infect. Immun.*, **65**, 4258–4266.
[160] Villeneuve, C., Gilleron, M., Maridonneau-Parini, I. *et al*. (2005) Mycobacteria use their surface-exposed glycolipids to infect human macrophages through a receptor-dependent process, *J. Lipid Res.*, **46**, 475–483.
[161] Armstrong, J.A. and Hart, P.D. (1975) Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual nonfusion pattern and observations on bacterial survival, *J. Exp. Med.*, **142**, 1–16.
[162] Basu, S. and Fenton, M.J. (2004) Toll-like receptors: function and roles in lung disease, *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **286**, L887–L892.

[163] Pandey, S., Kawai, T. and Akira, S. (2014) Microbial Sensing by Toll-Like Receptors and Intracellular Nucleic Acid Sensors, *Cold Spring Harbor Perspect. Biol.*, **7**, a016246–a016246.
[164] Kawasaki, T. and Kawai, T. (2014) Toll-like receptor signaling pathways, *Front. Immunol.*, **5**, 461.
[165] Kawai, T. and Akira, S. (2011) Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity, *Immunity*, **34**, 637–650.
[166] Cambi, A., Koopman, M. and Figdor, C.G. (2005) How C-type lectins detect pathogens, *Cell. Microbiol.*, **7**, 481–488.
[167] Poltorak, A., He, X., Smirnova, I. *et al*. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene, *Science*, **282**, 2085–2088.
[168] Malhotra, R., Thiel, S., Reid, K.B.M. and Sim, R.B. (1990) Human leukocyte C1q receptor binds other soluble proteins with collagen domains, *J. Exp. Med.*, **172**, 955–959.
[169] Akira, S., Uematsu, S. and Takeuchi, O. (2006) Pathogen recognition and innate immunity, *Cell*, **124**, 783–801.
[170] Yamamoto, M., Takeda, K. and Akira, S. (2004) TIR domain-containing adaptors define the specificity of TLR signaling, *Mol. Immunol.*, **40**, 861–868.
[171] Kobayashi, K., Hernandez, L.D., Galan, J.E. *et al*. (2002) IRAK-M is a negative regulator of Toll-like receptor signaling, *Cell*, **110**, 191–202.
[172] Means, T.K., Lien, E., Yoshimura, A. *et al*. (1999) The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors, *J. Immunol.*, **163**, 6748–6755.
[173] Jones, B.W., Means, T.K., Heldwein, K.A. *et al*. (2001) Different Toll-like receptor agonists induce distinct macrophage responses, *J. Leukoc. Biol.*, **69**, 1036–1044.
[174] Kindrachuk, J., Potter, J., Wilson, H.L. *et al*. (2008) Activation and regulation of toll-like receptor 9: CpGs and beyond, *Mini Rev. Med. Chem.*, **8**, 590–600.
[175] Báfica, A., Scanga, C.A., Feng, C.G. *et al*. (2005) TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis, *J. Exp. Med.*, **202**, 1715–1724.
[176] Drennan, M.B., Nicolle, D., Quesniaux, V.J. *et al*. (2004) Toll-like receptor 2-deficient mice succumb to Mycobacterium tuberculosis infection, *Am. J. Pathol.*, **164**, 49–57.
[177] Abel, B., Thieblement, N., Quesniaux, V.J.F. *et al*. (2002) Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice, *J. Immunol.*, **169**, 3155–3162.
[178] Reiling, N., Holscher, C., Fehrenbach, A. *et al*. (2002) Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis, *J. Immunol.*, **169**, 3480–3484.
[179] Hölscher, C., Reiling, N., Schaible, U.E. *et al*. (2008) Containment of aerogenic Mycobacterium tuberculosis infection in mice does not require MyD88 adaptor function for TLR2, -4 and -9, *Eur. J. Immunol.*, **38**, 680–694.
[180] Fremond, C.M., Yeremeev, V., Nicolle, D.M. *et al*. (2004) Fatal Mycobacterium tuberculosis infection despite adaptive immune response in the absence of MyD88, *J. Clin. Invest.*, **114**, 1790–1799.
[181] Canton, J., Neculai, D. and Grinstein, S. (2013) Scavenger receptors in homeostasis and immunity, *Nat. Rev. Immunol.*, **13**, 621–634.
[182] Doi, T., Higashino, K. I., Kurihara, *et al*. (1993) Charged collagen structure mediates the recognition of negatively charged macromolecules by macrophage scavenger receptors, *J. Biol. Chem.*, **268**, 2126–2133.
[183] Yamamoto, K., Nishimura, N., Doi, T. *et al*. (1997) The lysine cluster in the collagen-like domain of the scavenger receptor provides for its ligand binding and ligand specificity, *FEBS Lett.*, **414**, 182–186.
[184] Arredouani, M.S., Palecanda, A., Koziel, H. *et al*. (2005) MARCO is the major binding receptor for unopsonized particles and bacteria on human alveolar macrophages, *J. Immunol.*, **175**, 6058–6064.

[185] Reddy, R.C. (2008) Immunomodulatory role of PPAR-gamma in alveolar macrophages, *J. Invest. Med.*, **56**, 522–527.
[186] Zimmerli, S., Edwards, S. and Ernst, J.D. (1996) Selective receptor blockade during phagocytosis does not alter the survival and growth of Mycobacterium tuberculosis in human macrophages, *Am. J. Respir. Cell Mol. Biol.*, **15**, 760–770.
[187] Bowdish, D.M.E., Sakamoto, K., Kim, M.J. *et al*. (2009) MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and Mycobacterium tuberculosis, *PLoS Pathog.*, **5**, e1000474.
[188] Mahajan, S., Dkhar, H.K., Chandra, V. *et al*. (2012) Mycobacterium tuberculosis modulates macrophage lipid-sensing nuclear receptors PPARγ and TR4 for survival, *J. Immunol.*, **188**, 5593–5603.
[189] Almeida, P.E., Roque, N.R., Magalh, E.S. *et al*. (2014) Differential TLR2 downstream signaling regulates lipid metabolism and cytokine production triggered by Mycobacterium bovis BCG infection, *BBA - Mol. Cell Biol. Lipids*, **1841**, 97–107.
[190] Asada, K., Sasaki, S., Suda, T. *et al*. (2004) Antiinflammatory Roles of Peroxisome Proliferator–activated Receptor γ in Human Alveolar Macrophages, *Am. J. Respir. Crit. Care Med.*, **169**, 195–200.
[191] Court, N., Vasseur, V., Vacher, R. *et al*. (2010) Partial Redundancy of the Pattern Recognition Receptors, Scavenger Receptors, and C-Type Lectins for the Long-Term Control of Mycobacterium tuberculosis Infection, *J. Immunol.*, **184**, 7057–7070.
[192] Arredouani, M.S., Yang, Z., Imrich, A. *et al*. (2006) The macrophage scavenger receptor SR-AI/II and lung defense against pneumococci and particles, *Am. J. Respir. Cell Mol. Biol.*, **35**, 474–478 (2006).
[193] Hollifield, M., Ghanem, E.B., De Villiers, W.J.S. and Garvy, B.A. (2007) Scavenger receptor A dampens induction of inflammation in response to the fungal pathogen Pneumocystis carinii, *Infect. Immun.*, **75**, 3999–4005.
[194] Arredouani, M., Yang, Z., Ning, Y. *et al*. (2004) The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles, *J. Exp. Med.*, **200**, 267–272.
[195] Hawkes, M., Li, X., Crockett, M. *et al*. (2010) CD36 deficiency attenuates experimental mycobacterial infection, *BMC Infect. Dis.*, **10**, 299.
[196] Flannagan, R.S., Jaumouillé, V. and Grinstein, S. (2012) The cell biology of phagocytosis, *Annu. Rev. Pathol.*, **7**, 61–98.
[197] Russell, D.G. (2001) Mycobacterium tuberculosis: here today, and here tomorrow, *Nat. Rev. Mol. Cell Biol.*, **2**, 569–577.
[198] Reiner, N.E. (1994) Altered cell signaling and mononuclear phagocyte deactivation during intracellular infection, *Immunol. Today*, **15**, 374–381.
[199] Deretic, V., Singh, S., Master, S. *et al*. (2006) Mycobacterium tuberculosis inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism, *Cell. Microbiol.*, **8**, 719–727 (2006).
[200] Lugo-Villarino, G. and Neyrolles, O. (2014) Manipulation of the Mononuclear Phagocyte System by Mycobacterium tuberculosis, *Cold Spring Harbor Perspect. Med.*, **4**, a018549.
[201] Shukla, S., Richardson, E.T., Athman, J.J. *et al*. (2014) Mycobacterium tuberculosis lipoprotein LprG binds lipoarabinomannan and determines its cell envelope localization to control phagolysosomal fusion, *PLoS Pathog.*, **10**, e1004471.
[202] Gaur, R.L., Ren, K., Blumenthal, A. *et al*. (2014) LprG-Mediated Surface Expression of Lipoarabinomannan Is Essential for Virulence of Mycobacterium tuberculosis, *PLoS Pathog.*, **10**, e1004376.
[203] Welin, A. and Lerm, M. (2012) Inside or outside the phagosome? The controversy of the intracellular localization of Mycobacterium tuberculosis, *Tuberculosis*, **92**, 113–120.
[204] Leake, E.S., Myrvik, Q.N. and Wright, M.J. (1984) Phagosomal membranes of Mycobacterium bovis BCG-immune alveolar macrophages are resistant to disruption by Mycobacterium tuberculosis H37Rv, *Infect. Immun.*, **45**, 443–446.

[205] McDonough, K.A., Kress, Y. and Bloom, B.R. (1993) Pathogenesis of tuberculosis: interaction of Mycobacterium tuberculosis with macrophages, *Infect. Immun.*, **61**, 2763–2773.
[206] Myrvik, Q.N., Leake, E.S. and Wright, M.J. (1984) Disruption of phagosomal membranes of normal alveolar macrophages by the H37Rv strain of Mycobacterium tuberculosis. A correlate of virulence, *Am. Rev. Respir. Dis.*, **129**, 322–328.
[207] Simeone, R., Bobard, A., Lippmann, J. *et al.* (2012) Phagosomal rupture by Mycobacterium tuberculosis results in toxicity and host cell death, *PLoS Pathog.*, **8**, e1002507.
[208] Van der Wel, N., Hava, D., Houben, D. *et al.* (2007) M tuberculosis and M leprae translocate from the phagolysosome to the cytosol in myeloid cells, *Cell*, **129**, 1287–1298.
[209] Houben, D., Demangel, C., van Ingen, J. *et al.* (2012) ESX-1-mediated translocation to the cytosol controls virulence of mycobacteria, *Cell. Microbiol.*, **14**, 1287–1298.
[210] Manzanillo, P.S., Shiloh, M.U., Portnoy, D.A. and Cox, J.S. *et al.* (2012) Mycobacterium tuberculosis activates the DNA-dependent cytosolic surveillance pathway within macrophages, *Cell Host Microbe*, **11**, 469–480.
[211] Watson, R.O., Manzanillo, P.S. and Cox, J.S. (2012) Extracellular M tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway, *Cell*, **150**, 803–815.
[212] Shen, H.-M. and Mizushima, N. (2014) At the end of the autophagic road: an emerging understanding of lysosomal functions in autophagy, *Trends Biochem. Sci.*, **39**, 61–71.
[213] Mizushima, N. and Komatsu, M. (2011) Autophagy: Renovation of Cells and Tissues, *Cell*, **147**, 728–741.
[214] Levine, B., Mizushima, N. and Virgin, H.W. (2011) Autophagy in immunity and inflammation, *Nature*, **469**, 323–335.
[215] Yang, C.S., Kim, J.J., Lee, H.M. *et al.* (2014) The AMPK-PPARGC1A pathway is required for antimicrobial host defense through activation of autophagy, *Autophagy*, **10**, 785–802.
[216] Abnave, P., Mottola, G., Gimenez, G. *et al.* (2014) Screening in Planarians Identifies MORN2 as a Key Component in LC3-Associated Phagocytosis and Resistance to Bacterial Infection, *Cell Host Microbe*, **16**, 338–350.
[217] Romagnoli, A., Etna, M.P., Giacomini, E. *et al.* (2012) ESX-1 dependent impairment of autophagic flux by Mycobacterium tuberculosis in human dendritic cells, *Autophagy*, **8**, 1357–1370.
[218] Shin, D.M., Jeon, B.Y., Lee, H.M. *et al.* (2010) Mycobacterium tuberculosis eis regulates autophagy, inflammation, and cell death through redox-dependent signaling, *PLoS Pathog.*, **6**, e1001230.
[219] Gutierrez, M.G., Master, S.S., Singh, S.B. *et al.* (2004) Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages, *Cell*, **119**, 753–766.
[220] Fabri, M., Stenger, S., Shin, D.-M. *et al.* (2011) Vitamin D Is Required for IFN-γ-Mediated Antimicrobial Activity of Human Macrophages, *Sci. Translat. Med.*, **3**, 104ra102.
[221] Castillo, E.F., Dekonenko, A., Arko-Mensah, J. *et al.* (2012) Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation, *Proc. Natl Acad. Sci. USA*, **109**, E3168–E3176.
[222] Kim, J.J., Lee, H.M., Shin, D.M. *et al.* (2012) Host cell autophagy activated by antibiotics is required for their effective antimycobacterial drug action, *Cell Host Microbe*, **11**, 457–468.
[223] Bento, C.F., Empadinhas, N. and Mendes, V. (2015) Autophagy in the Fight Against Tuberculosis, *DNA Cell Biol.*, 150127063127004–150127063127015.
[224] Stanley, S.A., Barczak, A.K., Silvis, M.R., *et al.* (2014) Identification of Host-Targeted Small Molecules That Restrict Intracellular Mycobacterium tuberculosis Growth, *PLoS Pathog.*, **10**, e1003946.
[225] Hu, D., Wu, J., Zhang, R. *et al.* (2014) Autophagy-targeted vaccine of LC3–LpqH DNA and its protective immunity in a murine model of tuberculosis, *Vaccine*, **32**, 2308–2314.
[226] Olive, A.J. and Sassetti, C.M. (2015) New TB treatments hiding in plain sight, *EMBO Mol. Med.*, **7**, 125–126.

[227] Ting, J.P.-Y., Duncan, J.A. and Lei, Y. (2010) How the noninflammasome NLRs function in the innate immune system, *Science*, **327**, 286–290.
[228] Franchi, L., Eigenbrod, T., Muñoz-Planillo, R. and Nuñez, G. *et al*. (2009) The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis, *Nat. Immunol.*, **10**, 241–247.
[229] Werner, J.L. and Steele, C. (2014) Innate Receptors and Cellular Defense against Pulmonary Infections, *J. Immunol.*, **193**, 3842–3850.
[230] Killick, K.E.N., Cheallaigh, C., O'Farrelly, C. *et al*. (2013) Receptor-mediated recognition of mycobacterial pathogens, *Cell. Microbiol.*, **15**, 1484–1495.
[231] Dos Santos, G., Kutuzov, M.A. and Ridge, K.M. (2012) The inflammasome in lung diseases, *AJP: Lung Cell. Mol. Physiol.*, **303**, L627–L633.
[232] Hansen, J.M., Golchin, S.A., Veyrier, F.J. *et al*. (2014) N-glycolylated peptidoglycan contributes to the immunogenicity but not pathogenicity of Mycobacterium tuberculosis, *J. Infect. Dis.*, **209**, 1045–1054.
[233] Coulombe, F., Divangahi, M., Veyrier, F. *et al*. (2009) Increased NOD2-mediated recognition of N-glycolyl muramyl dipeptide, *J. Exp. Med.*, **206**, 1709–1716.
[234] Inohara, N., Ogura, Y., Fontalba, A. *et al*. (2003) Host recognition of bacterial muramyl dipeptide mediated through NOD2 Implications for Crohn's disease, *J. Biol. Chem.*, **278**, 5509–5512.
[235] Brooks, M.N., Rajaram, M.V.S., Azad, A.K. *et al*. (2011) NOD2 controls the nature of the inflammatory response and subsequent fate of Mycobacterium tuberculosis and M bovis BCG in human macrophages, *Cell. Microbiol.*, **13**, 402–418.
[236] Divangahi, M., Mostowy, S., Coulombe, F. *et al*. (2008) NOD2-deficient mice have impaired resistance to Mycobacterium tuberculosis infection through defective innate and adaptive immunity, *J. Immunol.*, **181**, 7157–7165.
[237] Gandotra, S., Jang, S., Murray, P.J. *et al*. (2007) Nucleotide-binding oligomerization domain protein 2-deficient mice control infection with Mycobacterium tuberculosis, *Infect. Immun.*, **75**, 5127–5134.
[238] Juárez, E., Carranza, C., Hernández-Sánchez, F. *et al*. (2012) NOD2 enhances the innate response of alveolar macrophages to Mycobacterium tuberculosis in humans, *Eur. J. Immunol.*, **42**, 880–889.
[239] McElvania Tekippe, E., Allen, I.C., Hulseberg, P.D. *et al*. (2010) Granuloma Formation and Host Defense in Chronic Mycobacterium tuberculosis Infection Requires PYCARD/ASC but Not NLRP3 or Caspase-1, *PLoS ONE*, **5**, e12320.
[240] Shah, S., Bohsali, A., Ahlbrand, S.E. *et al*. (2013) Cutting edge: Mycobacterium tuberculosis but not nonvirulent mycobacteria inhibits IFN-β and AIM2 inflammasome-dependent IL-1β production via its ESX-1 secretion system, *J. Immunol.*, **191**, 3514–3518.
[241] Saiga, H., Kitada, S., Shimada, Y. *et al*. (2012) Critical role of AIM2 in Mycobacterium tuberculosis infection, *Int. Immunol.*, **24**, 637–644.
[242] Glass, C.K. and Ogawa, S. (2006) Combinatorial roles of nuclear receptors in inflammation and immunity, *Nat. Rev. Immunol.*, **6**, 44–55.
[243] Ahmadian, M., Suh, J.M., Hah, N. *et al*. (2013) PPARγ signaling and metabolism: the good, the bad and the future, *Nat. Med.*, **19**, 557–566.
[244] Malur, A., Mccoy, A.J., Arce, S. *et al*. (2009) Deletion of PPAR gamma in Alveolar Macrophages Is Associated with a Th-1 Pulmonary Inflammatory Response, *J. Immunol.*, **182**, 5816–5822.
[245] Almeida, P.E., Silva, A.R., Maya-Monteiro, C.M. *et al*. (2009) Mycobacterium bovis bacillus Calmette-Guérin infection induces TLR2-dependent peroxisome proliferator-activated receptor gamma expression and activation: functions in inflammation, lipid metabolism, and pathogenesis, *J. Immunol.*, **183**, 1337–1345.
[246] Guo, H., Ingolia, N.T., Weissman, J.S. and Bartel, D.P. (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels, *Nature*, **466**, 835–840.
[247] Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function, *Cell*, **116**, 281–297.

[248] He, L. and Hannon, G.J. (2004) MicroRNAs: small RNAs with a big role in gene regulation, *Nat. Rev. Genet.*, **5**, 522–531.
[249] Foster, P.S., Plank, M., Collison, A. *et al*. (2013) The emerging role of microRNAs in regulating immune and inflammatory responses in the lung, *Immunol. Rev.*, **253**, 198–215.
[250] Sittka, A. and Schmeck, B. (2013) MicroRNAs in the lung, *Adv. Exp. Med. Biol.*, **774**, 121–134.
[251] Ma, C., Li, Y., Li, M. *et al*. (2014) microRNA-124 negatively regulates TLR signaling in alveolar macrophages in response to mycobacterial infection, *Mol. Immunol.*, **62**, 150–158.
[252] Kumar, M., Sahu, S.K., Kumar, R. *et al*. (2015) MicroRNA let-7 Modulates the Immune Response to Mycobacterium tuberculosis Infection via Control of A20, an Inhibitor of the NF-kappaB Pathway, *Cell Host Microbe*, 1–13.
[253] Ni, B., Rajaram, M.V.S., Lafuse, W.P. *et al*. (2014) Mycobacterium tuberculosis decreases human macrophage IFN-γ responsiveness through miR-132 and miR-26a, *J. Immunol.*, **193**, 4537–4547.
[254] Dorhoi, A., Iannaccone, M., Farinacci, M. *et al*. (2013) MicroRNA-223 controls susceptibility to tuberculosis by regulating lung neutrophil recruitment, *J. Clin. Invest.*, **123**, 4836–4848.
[255] Liu, Y., Wang, X., Jiang, J. *et al*. (2011) Modulation of T cell cytokine production by miR-144* with elevated expression in patients with pulmonary tuberculosis, *Mol. Immunol.*, **48**, 1084–1090.
[256] Ma, F., Xu, S., Liu, X. *et al*. (2011) The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-γ, *Nat. Immunol.*, **12**, 861–869.
[257] Yi, Z., Fu, Y., Ji, R. *et al*. (2012) Altered microRNA signatures in sputum of patients with active pulmonary tuberculosis, *PLoS ONE*, **7**, e43184.
[258] Singh, Y., Kaul, V., Mehra, A. *et al*. (2013) Mycobacterium tuberculosis controls microRNA-99b (miR-99b) expression in infected murine dendritic cells to modulate host immunity, *J. Biol. Chem.*, **288**, 5056–5061.
[259] Wang, J., Yang, K., Zhou, L. *et al*. (2013) MicroRNA-155 promotes autophagy to eliminate intracellular mycobacteria by targeting Rheb, *PLoS Pathog.*, **9**, e1003697.
[260] Rajaram, M.V.S., Ni, B., Morris, J.D. *et al*. (2011) Mycobacterium tuberculosis lipomannan blocks TNF biosynthesis by regulating macrophage MAPK-activated protein kinase 2 (MK2) and microRNA miR-125b, *Proc. Natl Acad. Sci. USA*, **108**, 17408–17413.
[261] Busch, S., Auth, E., Scholl, F. *et al*. (2015) 5-Lipoxygenase Is a Direct Target of miR-19a-3p and miR-125b-5p, *J. Immunol.*, **194**, 1646–1653.
[262] Iannaccone, M., Dorhoi, A. and Kaufmann, S.H.E. (2014) Host-directed therapy of tuberculosis: what is in it for microRNA?, *Expert Opin. Ther. Targets*, **18**, 491–494.
[263] Lamkanfi, M. and Dixit, V.M. (2010) Manipulation of Host Cell Death Pathways during Microbial Infections, *Cell Host Microbe*, **8**, 44–54.
[264] Duprez, L., Wirawan, E., Berghe, T.V. and Vandenabeele, P. (2009) Major cell death pathways at a glance, *Microbes Infect.*, **11**, 1050–1062.
[265] Golstein, P. and Kroemer, G. (2007) Cell death by necrosis: towards a molecular definition, *Trends Biochem. Sci.*, **32**, 37–43.
[266] Bergsbaken, T., Fink, S.L. and Cookson, B.T. (2009) Pyroptosis: host cell death and inflammation, *Nat. Rev. Microbiol.*, **7**, 99–109.
[267] Srinivasan, L., Ahlbrand, S. and Briken, V. (2014) Interaction of Mycobacterium tuberculosis with host cell death pathways, *Cold Spring Harbor Perspect Med.*, **4**, a022459.
[268] Behar, S.M., Martin, C.J., Booty, M.G. *et al*. (2011) Apoptosis is an innate defense function of macrophages against Mycobacterium tuberculosis, *Mucosal Immunol.*, **4**, 279–287.
[269] Briken, V. (2013) Mycobacterium tuberculosis genes involved in regulation of host cell death, *Adv. Exp. Med. Biol.*, **783**, 93–102.
[270] Geering, B. and Simon, H.-U. (2011) Peculiarities of cell death mechanisms in neutrophils, *Cell Death Differ.*, **18**, 1457–1469.
[271] Lowe, D.M., Redford, P.S., Wilkinson, R.J. *et al*. (2012) Neutrophils in tuberculosis: friend or foe?, *Trends Immunol.*, **33**, 14–25.

[272] Eum, S.Y., Kong, J.H., Hong, M.S. *et al*. (2010) Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB, *Chest*, **137**, 122–128.
[273] Martineau, A.R., Newton, S.M., Wilkinson, K.A. *et al*. (2007) Neutrophil-mediated innate immune resistance to mycobacteria, *J. Clin. Invest.*, **117**, 1988–1994.
[274] Berry, M.P.R., Graham, C.M., McNab, F.W. *et al*. (2010) An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis, *Nature*, **466**, 973–977.
[275] Seiler, P., Aichele, P., Raupach, B. *et al*. (2000) Rapid Neutrophil Response Controls Fast-Replicating Intracellular Bacteria but Not Slow-Replicating Mycobacterium tuberculosis, *J. Infect. Dis.*, **181**, 671–680.
[276] D'Avila, H., Roque, N.R., Cardoso, R.M. *et al*. (2008) Neutrophils recruited to the site of Mycobacterium bovis BCG infection undergo apoptosis and modulate lipid body biogenesis and prostaglandin E 2 production by macrophages, *Cell. Microbiol.*, **10**, 2589–2604.
[277] Abadie, V., Badell, E., Douillard, P. *et al*. (2005) Neutrophils rapidly migrate via lymphatics after Mycobacterium bovis BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes, *Blood*, **106**, 1843–1850.
[278] Seiler, P., Aichele, P., Bandermann, S. *et al*. (2003) Early granuloma formation after aerosol Mycobacterium tuberculosis infection is regulated by neutrophils via CXCR3-signaling chemokines, *Eur. J. Immunol.*, **33**, 2676–2686.
[279] Korbel, D.S., Schneider, B.E. and Schaible, U.E (2008) Innate immunity in tuberculosis: myths and truth, *Microbes Infect.*, **10**, 995–1004.
[280] Corleis, B., Korbel, D., Wilson, R. *et al*. (2012) Escape of Mycobacterium tuberculosis from oxidative killing by neutrophils, *Cell. Microbiol.*, **14**, 1109–1121.
[281] Reyes-Ruvalcaba, D., González-Cortés, C. and Rivero-Lezcano, O.M. (2008) Human phagocytes lack the ability to kill Mycobacterium gordonae, a non-pathogenic mycobacteria, *Immunol. Lett.*, **116**, 72–78.
[282] Ramos-Kichik, V., Mondragón-Flores, R., Mondragón-Castelán, M. *et al*. (2009) Neutrophil extracellular traps are induced by Mycobacterium tuberculosis, *Tuberculosis (Edinburgh)*, **89**, 29–37.
[283] Welin, A., Björnsdottir, H., Winther, M. *et al*. (2015) CFP-10 from Mycobacterium tuberculosis Selectively Activates Human Neutrophils through a Pertussis Toxin-Sensitive Chemotactic Receptor, *Infect. Immun.*, **83**, 205–213.
[284] Mócsai, A. (2013) Diverse novel functions of neutrophils in immunity, inflammation, and beyond, *J. Exp. Med.*, **210**, 1283–1299.
[285] Nandi, B. and Behar, S.M. (2011) Regulation of neutrophils by interferon-γ limits lung inflammation during tuberculosis infection, *J. Exp. Med.*, **208**, 2251–2262.
[286] Yang, C.T., Cambier, C.J., Davis, J.M. *et al*. (2012) Neutrophils Exert Protection in the Early Tuberculous Granuloma by Oxidative Killing of Mycobacteria Phagocytosed from Infected Macrophages, *Cell Host Microbe*, **12**, 301–312.
[287] Tailleux, L., Neyrolles, O., Honoré-Bouakline, S. *et al*. (2003) Constrained Intracellular Survival of Mycobacterium tuberculosis in Human Dendritic Cells, *J. Immunol.*, **170**, 1939–1948.
[288] Buettner, M., Meinken, C., Bastian, M. *et al*. (2005) Inverse Correlation of Maturity and Antibacterial Activity in Human Dendritic Cells, *J. Immunol.*, **174**, 4203–4209.
[289] Madan-Lala, R., Sia, J.K., King, R. *et al*. (2014) Mycobacterium tuberculosis Impairs Dendritic Cell Functions through the Serine Hydrolase Hip1, *J. Immunol.*, **192**, 4263–4272.
[290] Kopf, M., Schneider, C. and Nobs, S.P. (2015) The development and function of lung-resident macrophages and dendritic cells, *Nat. Immunol.*, **16**, 36–44.
[291] Reljic, R., Di Sano, C., Crawford, C. *et al*. (2005) Time course of mycobacterial infection of dendritic cells in the lungs of intranasally infected mice, *Tuberculosis (Edinburgh)*, **85**, 81–88.
[292] Lagranderie, M., Nahori, M.A., Balazuc, A.M. *et al*. (2003) Dendritic cells recruited to the lung shortly after intranasal delivery of Mycobacterium bovis BCG drive the primary immune response towards a type 1 cytokine production, *Immunology*, **108**, 352–364.
[293] Henderson, R.A., Watkins, S.C. and Flynn, J.L. (1997) Activation of human dendritic cells following infection with Mycobacterium tuberculosis, *J. Immunol.*, **159**, 635–643.

[294] Giacomini, E., Lona, E., Ferroni, L. *et al*. (2001) Infection of human macrophages and dendritic cells with Mycobacterium tuberculosis induces a differential cytokine gene expression that modulates T cell response, *J. Immunol.*, **166**, 7033–7041.
[295] Cooper, A.M., Solache, A. and Khader, S.A. (2007) Interleukin-12 and tuberculosis: an old story revisited, *Curr. Opin. Immunol.*, **19**, 441–447.
[296] Hickman, S.P., Chan, J. and Salgame, P. (2002) Mycobacterium tuberculosis Induces Differential Cytokine Production from Dendritic Cells and Macrophages with Divergent Effects on Naive T Cell Polarization, *J. Immunol.*, **168**, 4636–4642.
[297] Cooper, M.A., Fehniger, T.A. and Caligiuri, M.A. (2001) The biology of human natural killer-cell subsets, *Trends Immunol.*, **22**, 633–640.
[298] Fan, Y.-Y., Yang, B.-Y. and Wu, C.-Y. (2008) Phenotypically and functionally distinct subsets of natural killer cells in human PBMCs, *Cell Biol. Int.*, **32**, 188–197.
[299] Schierloh, P., Yokobori, N., Alemán, M. *et al*. (2005) Increased susceptibility to apoptosis of CD56dimCD16+ NK cells induces the enrichment of IFN-gamma-producing CD56bright cells in tuberculous pleurisy, *J. Immunol.*, **175**, 6852–6860.
[300] Molloy, A. (1994) Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin, *J. Exp. Med.*, **180**, 1499–1509.
[301] Vankayalapati, R., Garg, A., Porgador, A.*et al*. (2005) Role of NK Cell-Activating Receptors and Their Ligands in the Lysis of Mononuclear Phagocytes Infected with an Intracellular Bacterium, *J. Immunol.*, **175**, 4611–4617.
[302] Garg, A., Barnes, P.F., Porgador, A. *et al*. (2006) Vimentin Expressed on Mycobacterium tuberculosis-Infected Human Monocytes Is Involved in Binding to the NKp46 Receptor, *J. Immunol.*, **177**, 6192–6198.
[303] Newman, K.C. and Riley, E.M. (2007) Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens, *Nat. Rev. Immunol.*, **7**, 279–291.
[304] Esin, S., Batoni, G., Counoupas, C. *et al*. (2008) Direct Binding of Human NK Cell Natural Cytotoxicity Receptor NKp44 to the Surfaces of Mycobacteria and Other Bacteria, *Infect. Immun.*, **76**, 1719–1727.
[305] Marcenaro, E., Ferranti, B., Falco, M.*et al*. (2008) Human NK cells directly recognize Mycobacterium bovis via TLR2 and acquire the ability to kill monocyte-derived DC, *Int. Immunol.*, **20**, 1155–1167.
[306] Esin, S., Counoupas, C., Aulicino, A.*et al*. (2013) Interaction of Mycobacterium tuberculosis Cell Wall Components with the Human Natural Killer Cell Receptors NKp44 and Toll-Like Receptor 2, *Scand. J. Immunol.*, **77**, 460–469.
[307] Ferlazzo, G., Morandi, B., D'Agostino, A.*et al*. (2003) The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells, *Eur. J. Immunol.*, **33**, 306–313.
[308] Gerosa, F. (2002) Reciprocal Activating Interaction between Natural Killer Cells and Dendritic Cells, *J. Exp. Med.*, **195**, 327–333.
[309] Chiesa, M.D., Carlomagno, S., Ferlazzo, G. *et al*. (2003) The natural killer cell-mediated killing of autologous dendritic cells is confined to a cell subset expressing CD94/NKG2A, but lacking inhibitory killer Ig-like receptors, *Eur. J. Immunol.*, **33**, 1657–1666.
[310] Esin, S. and Batoni, G. (2015) Natural Killer Cells: A Coherent Model for Their Functional Role in Mycobacterium tuberculosis Infection, *J. Innate Immun.*, **7**, 11–24.
[311] Casadevall, A. (1998) Antibody-mediated protection against intracellular pathogens, *Trends Microbiol.*, **6**, 102–107.
[312] Balu, S., Reljic, R., Lewis, M.J. *et al*. (2011) A Novel Human IgA Monoclonal Antibody Protects against Tuberculosis, *J. Immunol.*, **186**, 3113–3119.
[313] Williams, A., Reljic, R., Naylor, I. *et al*. (2004) Passive protection with immunoglobulin A antibodies against tuberculous early infection of the lungs, *Immunology*, **111**, 328–333.
[314] Kunnath-Velayudhan, S., Salamon, H., Wang, H.Y.*et al*. (2010) Dynamic antibody responses to the Mycobacterium tuberculosis proteome, *Proc. Natl Acad. Sci. USA*, **107**, 14703–14708.
[315] Lugo-Villarino, G., Hudrisier, D., Benard, A. and Neyrolles, O. (2012) Emerging Trends in the Formation and Function of Tuberculosis Granulomas, *Front. Immunol.*, **3**, 405 (2012).

[316] Itano, A.A. and Jenkins, M.K. (2003) Antigen presentation to naive CD4 T cells in the lymph node, *Nat. Immunol.*, **4**, 733–739.
[317] Townsend, S.E. and Goodnow, C.C. (1998) Abortive Proliferation of Rare T Cells Induced by Direct or Indirect Antigen Presentation by Rare B Cells In Vivo, *J. Exp. Med.*, **187**, 1611–1621.
[318] Kozakiewicz, L., Chen, Y., Xu, J. *et al*. (2013) B Cells Regulate Neutrophilia during Mycobacterium tuberculosis Infection and BCG Vaccination by Modulating the Interleukin-17 Response, *PLoS Pathog.*, **9**, e1003472.
[319] Maglione, P.J., Xu, J. and Chan, J. (2007) B Cells Moderate Inflammatory Progression and Enhance Bacterial Containment upon Pulmonary Challenge with Mycobacterium tuberculosis, *J. Immunol.*, **178**, 7222–7234.
[320] Maglione, P.J., Xu, J., Casadevall, A. and Chan, J. (2008) Fcγ Receptors Regulate Immune Activation and Susceptibility during Mycobacterium tuberculosis Infection, *J. Immunol.*, **180**, 3329–3338.
[321] Bosio, C.M., Gardner, D. and Elkins, K.L. (2000) Infection of B Cell-Deficient Mice with CDC 1551, a Clinical Isolate of Mycobacterium tuberculosis: Delay in Dissemination and Development of Lung Pathology, *J. Immunol.*, **164**, 6417–6425.
[322] Torrado, E., Fountain, J.J., Robinson, R.T. *et al*. (2013) Differential and Site Specific Impact of B Cells in the Protective Immune Response to Mycobacterium tuberculosis in the Mouse, *PLoS ONE*, **8** e61681.
[323] Reiley, W.W., Calayag, M.D., Wittmer, S.T. *et al*. (2008) ESAT-6-specific CD4 T cell responses to aerosol Mycobacterium tuberculosis infection are initiated in the mediastinal lymph nodes, *Proc. Natl Acad. Sci. USA*, **105**, 10961–10966.
[324] Chackerian, A.A., Alt, J.M., Perera, T.V. *et al*. (2002) Dissemination of Mycobacterium tuberculosis is influenced by host factors and precedes the initiation of T-cell immunity, *Infect. Immun.*, **70**, 4501–4509.
[325] Casanova, J.-L. and Abel, l. (2002) Genetic dissection of immunity to mycobacteria: the human model, *Annu. Rev. Immunol.*, **20**, 581–620.
[326] Flynn, J.L., Chan, J., Triebold, K.J. *et al*. (1993) An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection, *J. Exp. Med.*, **178**, 2249–2254.
[327] Caruso, A.M., Serbina, N., Klein, E.*et al*. (1999) Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis, *J. Immunol.*, **162**, 5407–5416.
[328] Gallegos, A.M., Van Heijst, J.W.J., Samstein, M. *et al*. (2011) A gamma interferon independent mechanism of CD4 T cell mediated control of M tuberculosis infection in vivo, *PLoS Pathog.*, **7** e1002052.
[329] Cowley, S.C. and Elkins, K.L. (2003) CD4+ T cells mediate IFN-gamma-independent control of Mycobacterium tuberculosis infection both in vitro and in vivo, *J. Immunol.*, **171**, 4689–4699.
[330] Woodworth, J.S., Wu, Y. and Behar, S.M. (2008) Mycobacterium tuberculosis-Specific CD8(+) T Cells Require Perforin to Kill Target Cells and Provide Protection In Vivo, *J. Immunol.*, **181**, 8595–8603.
[331] Sousa, A.O., Mazzaccaro, R.J., Russell, R.G. *et al*. (2000) Relative contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice, *Proc. Natl Acad. Sci. USA*, **97**, 4204–4208.
[332] Behar, S.M., Dascher, C.C., Grusby, M.J. *et al*. (1999) Susceptibility of Mice Deficient in CD1D or TAP1 to Infection with Mycobacterium tuberculosis, *J. Exp. Med.*, **189**, 1973–1980.
[333] Ryan, A.A., Nambiar, J.K., Wozniak, T.M. *et al*. (2009) Antigen Load Governs the Differential Priming of CD8 T Cells in Response to the Bacille Calmette Guerin Vaccine or Mycobacterium tuberculosis Infection, *J. Immunol.*, **182**, 7172–7177.
[334] Gold, M.C., Napier, R.J. and Lewinsohn, D.M. (2015) MR1-restricted mucosal associated invariant T (MAIT) cells in the immune response to Mycobacterium tuberculosis, *Immunol. Rev.*, **264**, 154–166.
[335] Le Bourhis, L., Martin, E., Péguillet, I. *et al*. (2010) Antimicrobial activity of mucosal-associated invariant T cells, *Nat. Immunol.*, **11**, 701–708.

[336] Chua, W.J., Truscott, S.M., Eickhoff, C.S. *et al*. (2012) Polyclonal mucosa-associated invariant T cells have unique innate functions in bacterial infection, *Infect. Immun.*, **80**, 3256–3267.
[337] Moody, D.B., Ulrichs, T., Mühlecker, W. *et al*. (2000) CD1c-mediated T-cell recognition of isoprenoid glycolipids in Mycobacterium tuberculosis infection, *Nature*, **404**, 884–888.
[338] Ulrichs, T., Moody, D.B., Grant, E. *et al*. (2003) T-Cell Responses to CD1-Presented Lipid Antigens in Humans with Mycobacterium tuberculosis Infection, *Infect. Immun.*, **71**, 3076–3087.
[339] Meraviglia, S., Caccamo, N., Salerno, A. *et al*. (2010) Partial and Ineffective Activation of Vγ9Vδ2 T Cells by Mycobacterium tuberculosis-Infected Dendritic Cells, *J. Immunol.*, **185**, 1770–1776.
[340] Martino, A., Casetti, R., Sacchi, A. and Poccia, F. (2007) Central Memory Vγ9Vδ2 T Lymphocytes Primed and Expanded by Bacillus Calmette-Guérin-Infected Dendritic Cells Kill Mycobacterial-Infected Monocytes, *J. Immunol.*, **179**, 3057–3064.
[341] Dieli, F., Sireci, G., Caccamo, N. *et al*. (2002) Selective depression of interferon-gamma and granulysin production with increase of proliferative response by Vgamma9/Vdelta2 T cells in children with tuberculosis, *J. Infect. Dis.*, **186**, 1835–1839.
[342] Shafiani, S., Dinh, C., Ertelt, J. *et al*. (2013) Pathogen-specific Treg cells expand early during mycobacterium tuberculosis infection but are later eliminated in response to Interleukin-12, *Immunity*, **38**, 1261–1270.
[343] Kursar, M., Koch, M., Mittrücker, H.-W. *et al*. (2007) Cutting Edge: Regulatory T Cells Prevent Efficient Clearance of Mycobacterium tuberculosis, *J. Immunol.*, **178**, 2661–2665.
[344] Boussiotis, V.A., Tsai, E.Y., Yunis, E.J. *et al*. (2000) IL-10-producing T cells suppress immune responses in anergic tuberculosis patients, *J. Clin. Invest.*, **105**, 1317–1325.
[345] Ramakrishnan, L. (2012) Revisiting the role of the granuloma in tuberculosis, *Nat. Rev. Immunol.*, **12**, 352–366.
[346] Tsai, M.C., Chakravarty, S., Zhu, G. *et al*. (2006) Characterization of the tuberculous granuloma in murine and human lungs: cellular composition and relative tissue oxygen tension, *Cell. Microbiol.*, **8**, 218–232.
[347] Guirado, E. and Schlesinger, L.S. (2013) Modeling the Mycobacterium tuberculosis Granuloma - the Critical Battlefield in Host Immunity and Disease, *Front. Immunol.*, **4**, 98.
[348] Kaplan, G., Post, F.A., Moreira, A.L. *et al*. (2003) Mycobacterium tuberculosis growth at the cavity surface: a microenvironment with failed immunity, *Infect. Immun.*, **71**, 7099–7108.
[349] Orme, I.M. and Basaraba, R.J. (2014) The formation of the granuloma in tuberculosis infection, *Semin. Immunol.*, **26**, 601–609.
[350] Russell, D.G. (2007) Who puts the tubercle in tuberculosis?, *Nat. Rev. Microbiol.*, **5**, 39–47.
[351] Silva Miranda, M., Breiman, A., Allain, S. *et al*. (2012) The Tuberculous Granuloma: An Unsuccessful Host Defence Mechanism Providing a Safety Shelter for the Bacteria?, *Clin. Dev. Immunol.*, **2012**, 1–14.
[352] Paige, C. and Bishai, W.R. (2010) Penitentiary or penthouse condo: the tuberculous granuloma from the microbe's point of view, *Cell. Microbiol.*, **12**, 301–309.
[353] Heitmann, L., Dar, M.A., Schreiber, T. *et al*. (2014) The IL-13/IL-4R αaxis is involved in tuberculosis-associated pathology, *J. Pathol.*, **234**, 338–350.
[354] Cyktor, J.C., Carruthers, B., Kominsky, R.A. *et al*. (2013) IL-10 Inhibits Mature Fibrotic Granuloma Formation during Mycobacterium tuberculosis Infection, *J. Immunol.*, **190**, 2778–2790.
[355] Pan, H., Yan, B.-S., Rojas, M. *et al*. (2005) Ipr1 gene mediates innate immunity to tuberculosis, *Nature*, **434**, 767–772.
[356] Manabe, Y.C., Kesavan, A.K., Lopez-Molina, J. *et al*. (2008) The aerosol rabbit model of TB latency, reactivation and immune reconstitution inflammatory syndrome, *Tuberculosis*, **88**, 187–196.
[357] Scanga, C.A. and Flynn, J.L. (2014) Modeling Tuberculosis in Nonhuman Primates, *Cold Spring Harbor Perspect. Med.*, **4**, a018564.

[358] McMurray, D. (1994) Guinea Pig Model of Tuberculosis, *in* Tuberculosis: Pathogenesis, Protection, and Control, (ed. B. Bloom), ASM Press, Washington, DC.
[359] Guirado, E., Mbawuike, U., Keiser, T.L. *et al*. (2015) Characterization of Host and Microbial Determinants in Individuals with Latent Tuberculosis Infection Using a Human Granuloma Model, *MBio*, 6, e02537–14–13.
[360] Puissegur, M.P., Botanch, C., Duteyrat, J.L. *et al*. (2004) An in vitro dual model of mycobacterial granulomas to investigate the molecular interactions between mycobacteria and human host cells, *Cell. Microbiol.*, **6**, 423–433.
[361] Marino, S., Linderman, J.J. and Kirschner, D.E. (2010) A multifaceted approach to modeling the immune response in tuberculosis, *WIREs Syst. Biol. Med.*, **3**, 479–489.
[362] Marino, S., El-Kebir, M. and Kirschner, D. (2011) A hybrid multi-compartment model of granuloma formation and T cell priming in Tuberculosis, *J. Theor. Biol.*, **280**, 50–62.
[363] Bulut, Y., Michelsen, K.S., Hayrapetian, L., Naiki, Y., Spallek, R., Singh, M. and Arditi, M. (2005) Mycobacterium tuberculosis heat shock proteins use diverse toll-like receptor pathways to activate pro-inflammatory signals, *J. Biol. Chem.*, **280**, 20961–20967.

3

Animal Models of Tuberculosis

David N. McMurray PhD

Department of Microbial Pathogenesis and Immunology, College of Medicine, Texas A&M University Health Science Center

3.1 Introduction

Advances in the development and application of new vaccines and drugs for tuberculosis (TB) have depended upon the use of biologically relevant animal models. Over the past 40 years, our concept of what comprises an animal model of TB and how such models should be employed has evolved. Animal models have been extremely valuable for elucidating the fundamental mechanisms of the pathogen's virulence and of natural and vaccine-induced host resistance. In addition, models have been essential for screening the literally hundreds of novel vaccines and drugs which have been identified in research laboratories to determine which of the candidates were worthy of additional pre-clinical development. The requirements of an animal model to study basic mechanisms of disease pathogenesis are somewhat different to those of a model which might be used empirically to screen novel biologics for *in vivo* efficacy. Furthermore, given the multiplicity of animal models of TB currently in use, the question of standardization has been debated among researchers of both TB vaccines and drugs. This chapter will summarize the evolution of the current strategy for applying animal models to the study of TB, and highlight the comparative advantages and disadvantages of the major models with regard to their use in pre-clinical testing of vaccines and drugs.

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

3.2 What is an Animal Model of TB?

An animal model of TB might be defined as the assignment of individual choices among the experimental variables which define the infectious disease; for example, animal species and strain, challenge strain, challenge route, measures of infection and/or disease, time post-challenge at which measurements are made, and so on. If the model is to be used for the evaluation of vaccine-induced protection, then additional parameters that will have to be fixed are the vaccination route and dose, and the vaccination–challenge interval. If one arbitrarily assigns just three of the many choices for each of the variables listed above, the total combinatorial number of possible models that differ by at least one of the variables is very large (more than 20,000!) [1]. In a review of publications from laboratories working on TB vaccines over a 10-year period, every one of the laboratories was using a different model and many laboratories were using more than one model as defined above [2]. More recently, in a review of publications reporting the protective efficacy of novel TB vaccines, the authors reported that the variability between animal models being employed remains enormous. Studies reviewed used a total of more than 40 animal models that differed from each other in animal species (three), routes of vaccination (six), vaccination–challenge intervals (eleven), routes of virulent challenge (four), and challenge doses (six). [3]. Thus, every TB lab in the world appears to be using a different animal model which renders impossible the direct comparison of results obtained with the testing of drugs and vaccines.

Given the multiplicity of TB animals models employed globally, the fundamental question is: does it matter? In other words, if comparable results can be obtained with different models, then model heterogeneity may not be as big an issue as it might seem. To test that hypothesis, the laboratory Don Smith at the University of Wisconsin organized an experiment in which five different TB vaccines were formulated in Madison and sent to eight well-established international TB laboratories to be ranked for protective efficacy in a total of 21 different animal models. The results showed that none of the 21 models ranked the vaccines in the same order. What is worse, the best vaccine in some models was the worst vaccine in others [1, 4]. It was clear that the model *did matter* when it came to evaluating candidate TB vaccines.

However, it was possible that much of the variability between animal models in the cooperative experiment just described was due to uncontrolled and unapparent differences between the eight laboratories that participated. Therefore, the Smith lab conducted a second experiment to remove inter-lab bias. Five of the animal models used in the cooperative international study were replicated in Madison and those models were used to rank the protective efficacy of four experimental TB vaccines. The results were a mirror image of those reported in the international trial. None of the five animal models ranked the four vaccines in the same order. Further analysis revealed that every variable in the model (e.g., vaccination route and dose, challenge route and dose, etc.) exerted a significant influence on the apparent degree of protection afforded by the candidate TB vaccines [5].

If laboratories insist on using different models and the model clearly influences the results of the experiments, then what can be done to improve the comparability between laboratories that are testing TB vaccines and drugs? One suggestion was to define a so-called "rational" animal model in which the variables were selected to mimic what was known about human TB. Thus, a species would be selected because it faithfully reproduced human TB-like pathogenesis, vaccination would be conducted under conditions which

were feasible for human use, the animals would be challenged with a very low dose of virulent *Mycobacterium tuberculosis* by the aerosol route, and readouts would have relevance for protection against clinical disease [1].

A second approach was to promote the use of so-called "standardized" animal models in which the variables were fixed in consultation with an international group of experts in animal models of TB. The Animal Models Task Force of the WHO Immunology of Mycobacteria (IMMYC) Steering Committee worked for several years to define and promote the use of standardized models. In the US, the National Institutes of Health (NIH) implemented a standardized animal model approach to TB vaccine testing by funding a contract to two laboratories to test vaccines as a service to the vaccine developers in well-defined mouse and guinea pig models. Over more than 10 years, hundreds of vaccines were tested under these very controlled conditions and the data provided to the investigators [6]. In Europe, a similar centralized TB vaccine-testing program was implemented by the EU TB Vaccine Cluster and many vaccines were tested in a guinea pig model [7]. An important assumption underlying the standardized model approach was that different laboratories could obtain comparable results if they each used the standard model. That hypothesis was tested recently when three laboratories (two in the US, one in the UK) were asked to rank an identical panel of TB vaccines in essentially the same animal model (i.e., guinea pigs vaccinated by the same route and dose and challenged with a low dose of the same virulent strain of *M. tuberculosis* by the respiratory route). The results showed remarkable similarities between the laboratories in terms of both the ranking of the vaccines and quantitative measures of their protective efficacy [8].

In any discussion of animal models of TB, there is an "elephant in the room", namely, the fact that we cannot effectively model what we do not understand. The lack of a detailed understanding of the pathogenesis of human TB limits our ability to devise more biologically relevant models [9]. Current models mimic human TB in important, but incomplete, ways and improving them will await a better understanding of TB pathogenesis. For that, we will need better animal models. Thus, the field is trapped in a "catch-22" conundrum. In addition, most of the animal models focus on primary, pulmonary TB. New models are needed that approximate other important forms of TB disease, including endogenous reactivation TB, exogenous re-infection TB, cavitary TB, TB meningitis, persistent or latent TB, and TB/HIV co-infection. While some progress has been made in recent years, we will be forced to use imperfect models for the foreseeable future.

3.3 How are Animal Models of TB Used?

Animal models of TB are used for four fundamental purposes; (a) to elucidate virulence strategies of the pathogen; (b) to define the mechanisms of natural and vaccine-induced resistance; (c) to screen novel anti-TB drug candidates; and (d) to screen novel vaccines. Given the emphasis in this book on delivery systems for prevention and treatment of TB, I will not discuss the use of animal models in basic research into the nature of the host–pathogen interaction. Suffice it to say that a wealth of published literature based upon studies in mice, guinea pigs, rabbits and non-human primates (NHP) have defined a plethora of virulence genes and mechanisms and have begun to clarify the variables which influence the outcome of the struggle between the pathogen and the host [10–15]. As with the screening process for better TB vaccines described above, the search for virulence genes in

M. tuberculosis has been carried out in a centralized manner with core NIH funding for a few institutions. The strategy has been to test mutant and complemented strains for candidate virulence genes in more than one animal model (usually mice and guinea pigs) involving low-dose respiratory challenge. The value of this comparative approach has been demonstrated in experiments where the two models differed in their ability to reveal the role of unique mycobacterial genes in post-immune survival and persistence of the pathogen [16].

One of the observations which have come out of basic research into TB pathogenesis in recent years has a profound impact on the use of animal models to screen novel therapeutic drugs and vaccines. It is becoming clear that there are multiple, distinct mycobacterial populations in the TB patient [10, 11]. These discrete bacterial populations exist in diverse anatomical sites (both pulmonary and extrapulmonary) where they may be exposed to a variety of different environmental conditions. One implication of this observation is that these distinct populations of *M. tuberculosis* employ different metabolic strategies that may make them more or less susceptible to anti-TB drugs. Furthermore, the access of immune cells to the different foci of infection may vary, thus affecting the ability of a TB vaccine to control and eliminate the pathogen from all infected sites. Given the aforementioned, it is important that we use animal models that mimic the heterogeneous conditions under which the pathogen exists so that the success of novel TB drugs (and vaccines) can be evaluated properly. It is no longer possible to consider the animal model as nothing more than a "furry test tube" in TB drug testing. A much more precise definition of the targeted bacterial population(s) and an animal model in which those populations actually exist and can be monitored quantitatively are required for modern pre-clinical drug assessment [8, 11, 12].

One of the tacit assumptions which underpin the use of animal models in the pre-clinical evaluation of new TB drugs and vaccines is that the models employed will predict the activity of the drug or vaccine in humans. Recently, that hypothesis was tested when a novel TB drug combination involving moxifloxacin which was designed to shorten the treatment regimen, and a new TB vaccine which was designed to boost pre-existing BCG vaccine-induced protection were actually tested in Phase III and Phase IIb clinical trials, respectively. Both the drug combination and the booster vaccine had shown significant efficacy in multiple animal models. Unfortunately, neither the therapeutic combination [17] nor the booster vaccine [18] was shown to be as effective in humans as the animal data predicted. Not surprisingly, these unexpected results triggered a re-evaluation of the models used to screen TB drugs and vaccines [19]. Some experts have called into question the whole reliance on imperfect animal models in screening new TB drugs and vaccines; however, the reality is that some sort of triage process is essential to reduce the hundreds of candidates being produced by research laboratories around the world to a number which is manageable in the context of Phase I and II human trials. A more rational response is to use these disappointing results to refine the animal models so that they become more predictive of activity in humans.

3.4 TB Animal Models Currently Used for TB Drug and Vaccine Evaluation

Animal models of TB have been compared frequently over the past several years [9, 20–24]. The models are usually grouped by animal species, and the advantages and disadvantages of each are well-known to most scientists in the field. Some models offer the ability to manipulate and monitor the immune system in great detail (e.g., mice), while

others reproduce specific aspects of human TB pathogenesis more faithfully (e.g., cavitary lesions and meningitis in rabbits, granuloma histopathology in guinea pigs, clinical readouts in NHP, etc.). In the context of screening large numbers of novel TB drugs and vaccines, other issues become paramount (e.g., cost, Animal Biosafety Level – 3 [ABSL-3] space, etc.). The arguments over which model is "best" have been replaced by the realization that each model brings something unique and valuable to the table and that the application of the model ought to be matched to the advantages which each model offers or the specific scientific question being asked. The concept of comparative studies in multiple models has been embraced as a way to mitigate against the risk that one model may give misleading results while similar results obtained in multiple models provide a more compelling reason to proceed with the development of a TB drug or vaccine. In fact, many of the TB drugs and vaccines currently in human trials have been tested in multiple animal models.

Recent reviews have focused on the value (or lack thereof) of animal model data in making decisions about which vaccines should be taken forward for testing in humans. The lack of validated immunological correlates or biomarkers of protection has been highlighted as a fundamental impediment to the rational extrapolation of animal model data to humans [19, 25–27). There appears to be a lack of objective criteria for advancing TB vaccine candidates through the pipeline from pre-clinical development to Phase I, II and III clinical trials [25]. Several authors have pointed out the need for standardized approaches for the selection and advancement of vaccines, including the use of standardized animal models for pre-clinical testing [19, 25, 26]. One of the principal weaknesses in the current vaccine-selection strategy is the absence of head-to-head testing of multiple vaccine candidates in the same animal model [25, 26]. Vaccine developers are reluctant to have their favorite vaccine candidates tested directly against other candidates. One solution to this problem has been the involvement of organizations such as the Aeras Global TB Vaccine Foundation (now Aeras), which obtains vaccine candidates from individual scientists and has the capacity to perform head-to-head evaluations. In response to the recent failure of the MVA85A vaccine to boost immune responses in BCG-vaccinated infants, a critical assessment of the fundamental differences between the animal models and the human vaccinees was conducted [19]. Differences in study design, age of vaccinees, environmental variables (diet, exposure to other infectious agents, etc.), and the challenge strain, dose and exposure frequency were identified as possible contributors to the failure of multiple animal models to predict the vaccine failure in humans [19]. To counter some of these perceived weakness of vaccine testing in highly artificial, conventional small-animal models, some authors have promoted the use of models of "natural infection" with *M. bovis* in domestic (e.g., cattle, farmed deer) or wild animal reservoirs (e.g., badgers in the UK, brush-tail possums in New Zealand) for human TB vaccine testing [28].

A similar analysis of the value of animal models in TB drug screening has been carried out by the authors of several excellent reviews published in recent years. Most authors agree that pre-clinical testing in validated small-animal models is an essential step in the pathway from TB drug discovery to clinical trials [29–32]). Various mouse models have been the most widely used historically because they are well-characterized and economical [29–31]. Given the large numbers of animals employed in, and the length of, most TB drug-evaluation experiments, the issue of cost is paramount [30, 31]. Other models are also employed (e.g., guinea pig, rat, rabbit) as an alternative to, or to confirm the results obtained

in, mouse models. There are important differences in the nature of the lesions which are produced following infection of different experimental animal species with virulent *M. tuberculosis*, and these differences are likely to affect the *in vivo* activity of novel TB drugs [31]. A recent comprehensive analysis of the various *in vitro* and *in vivo* assays used historically and currently to evaluate TB drugs revealed a disturbing multiplicity of animal models. The authors pointed out the value of rationally-derived and -optimized models, and stressed the importance of confirmatory testing of TB drugs in different laboratories with different animal models to validate the results obtained under diverse experimental conditions [32]. As mentioned above, the challenge of treating a disease in which the tissues may contain multiple bacillary populations existing under different metabolic/growth conditions is daunting, since a particular animal model may not replicate faithfully the conditions under which tubercle bacilli exist in the human host [33, 34]. In particular, animal models such as the rabbit and non-human primate (NHP) which reproduce granuloma heterogeneity may allow investigators to apply modern imaging technologies to assess pharmacokinetic (PK) and pharmacodynamic (PD) properties of novel TB drugs in a "lesion-centric" fashion [34].

3.4.1 Guinea Pig

The guinea pig has served as an excellent model of human TB in the "susceptible" portion of the population who develop progressive disease upon exposure to a very low dose by the respiratory route. The basic characteristics of TB in guinea pigs have been described in previous reviews [17, 35–37]. A recent review highlighted the importance of the guinea pig model in TB vaccine and drug development as the focus of more than one-third of 550 publications on the model since 1982 [17]. The human-like granuloma structure and the ability to isolate and study discrete subpopulations of mycobacteria in the lungs and other tissues have been pointed out as distinct advantages of the model [38–41]. The impact of the local tissue environment (e.g., hypoxia) and the location (i.e., intracellular *vs* extracellular) and metabolic status of the pathogen exert a profound impact on the activity of some anti-mycobacterial drugs, and the guinea pig model allows such effects to be studied [38, 42]. These differences between the nature of the pulmonary lesions in mouse and guinea pig TB have been demonstrated to exert a significant influence on the apparent efficacy of some anti-TB drugs [39, 40]. The guinea pig may be superior at discriminating between purely bactericidal and sterilizing drugs, especially given the propensity of mycobacteria to persist within necrotic granulomas in that model where they may be extracellular [43–45].

The usefulness of the guinea pig model, especially in the pre-clinical development of new TB vaccines, has been limited by the lack of immunological reagents with which to elucidate the determinants on natural and vaccine-induced resistance to pulmonary TB. The availability and application of monoclonal antibodies to guinea pig cell surface antigens was reviewed recently [46]. Other publications have suggested that cross-reactive monoclonal antibodies directed against cell-associated and soluble immune molecules in other species (e.g., human, mouse) can be used to characterize the guinea pig immune response [47, 48]. The publication and subsequent annotation of the guinea pig genome (http://imap.broad.mit.edu/mailman/listinfo/caviaporcellusgenome; http://genome.ucsc.edu) has opened up an alternative strategy to the development of novel immunological

reagents for the guinea pig, namely, the cloning and expression of guinea pig genes which encode immune molecules. Over the past 10 years, several guinea pig cytokine genes have been cloned and expressed in prokaryotic and eukaryotic expression systems [49–58]. The resulting recombinant guinea pig proteins and polyclonal antibodies directed those proteins have been used to modulate cytokine functions in *ex vivo* cell culture systems [59–61]. In addition, the responses of vaccinated and/or infected guinea pigs have been altered by blocking or augmenting the activities of *cytokines in* vivo with these reagents [62–64]. Laser capture micro-dissection studies of cytokine mRNA expression *in situ* in guinea pig primary and secondary granulomas have revealed important insights into the local modulation of the immune response in vaccinated and non-vaccinated animals [65, 66]. The NIH has instituted a contract-funded mechanism by which guinea pig reagents can be developed and made available to the research community (www.beiresources.org). Such efforts will enhance the utility of the guinea pig model in the development and pre-clinical testing of novel TB drugs and vaccines.

3.4.2 Mouse

The mouse model has been the most widely used TB animal model for several obvious reasons [21]. The mouse immune response is the best characterized of any species and an almost unlimited array of immunological reagents is available commercially. The ability to manipulate the genetic repertoire of mouse strains by various knock-out, knock-in, and selective expression strategies affords a powerful means of dissecting the role of specific host factors in TB pathogenesis. The low cost and minimal ABSL-3 space requirements make the mouse the model of choice in TB vaccine and drug testing [21]. A number of recent publications highlight the usefulness of the mouse in the evaluation of novel TB drugs [67], including direct pulmonary delivery in insufflated powders [68], the efficacy of novel regimens against various strains of *M. tuberculosis* [69], and the comparison of drug responses in inbred mouse strains which differ in their TB disease presentation [70]. In a head-to-head study, the authors compared six different mouse models (three challenge routes X two inbred mouse strains) for their responses to a standard TB treatment regimen and found that model variables exerted a significant effect on the apparent efficacy of the regimen [71].

One perceived disadvantage of the mouse model is that the pulmonary granulomas do not mimic important characteristics of human TB granulomas, e.g., caseous necrosis, hypoxia, etc. [42]. Several years ago, a particular inbred mouse strain (C3HeB/FeJ) was observed to recapitulate human-like granulomas following infection with virulent *M. tuberculosis*, complete with hypoxia, necrosis and liquefaction [72–74]. That mouse strain has been used to evaluate novel anti-TB drugs which may target persistent bacilli residing in necrotic foci [74, 75]. Recently, the mouse has been manipulated to model forms of TB other than primary, pulmonary infection, including musculoskeletal TB [76] and reactivation TB [77]. Irradiated mice reconstituted with various human immune components (so-called "humanized" mice) have been used to study infectious diseases that are impossible to study because of their strict human host tropism (e.g., HIV, etc.) [78]. Humanized mice have also been used to study TB although it is still too early to appreciate the unique advantages that this model might offer [79, 80]. One obvious application is the study of TB/HIV co-infection that can otherwise only be investigated in NHP.

Two recent publications highlight the value of the mouse model in understanding the interplay between the host immune response and heterogeneous mycobacterial subpopulations within the lungs. In one study, the investigators measured single-cell fluorescence using an rRNA-GFP reporter system to demonstrate that chronically infected mice harbored a subpopulation of non-growing, but metabolically active, *M. tuberculosis* which were prominent in mice treated with isoniazid [81]. In another study, the investigators used reporter strains of *M. tuberculosis* that responded to specific host stresses such as hypoxia, nitric oxide, and phagosome maturation. Upon intranasal infection of mice, the reporter strains revealed a heterogeneous response to both vaccination and treatment of the mice with isoniazid [82]. Thus, while conventional mouse models may be somewhat limited with regard to investigating the compartmentalization of the host–pathogen interaction within discrete micro-foci, the mouse remains the TB model of choice for the application of cutting-edge technologies which may reveal novel aspects of pathogenesis that impact the efficacy of both vaccines and therapies.

3.4.3 Non-human Primate

The early application of NHP models to the study of the pathogenesis of pulmonary TB and response to vaccination and therapies consisted of several papers published between 1968 and 1975 that were reviewed [83]. Those studies involved Rhesus macaques infected with low doses of virulent *M. tuberculosis* by aerosol exposure or intra-bronchial instillation or insufflation. Following an interval of more than 20 years during which no studies of TB in NHP were published, the so-called "modern era" of that type of research was initiated in 1996 by the report of a study conducted in cynomolgus macaques [84], followed in 2001 by a study of BCG vaccination in NHP [85]. The unique advantages of the NHP model in terms of faithfully reproducing human-like disease have been summarized in two recent reviews [22, 86]. One of these papers includes a very complete time-line of the use of NHP in TB research and extensive tables summarizing the results of experiments published in the past 15 years [22]. Both reviews contain tables that compare the general features of the two principal species employed (Rhesus *vs* Cynomolgus macaques). Recently, a New World primate, the common marmoset, was reported to develop a very human-like disease following low-dose, aerogenic infection with several strains of *M. tuberculosis*. Two advantages of marmosets are their small size and ease of handling relative to the macaques [87].

In spite of the obvious impediments to the use of NHP in TB research (i.e., availability, cost, ABLS-3 space requirements, personnel safety, public sentiment), a significant amount of work has been carried out in the past 15 years to apply this model to the evaluation of novel vaccines and drugs [22, 86]. A rhesus macaque model was used to determine endpoints for vaccine-induced protection. The authors concluded that only the lung lesion burden as assessed by magnetic resonance imaging (MRI) combined with stereology on histopathological examination was shown to distinguish between vaccinated (BCG, or BCG+MVA85A) and non-vaccinated animals [88]. Radiological imaging was also reported to be useful in the assessment of TB chemotherapeutic regimens. Reduction in lesion size and activity as measured by positron emission tomography (PET) and computed tomography (CT) was associated with isoniazid therapy in cynomolgus macaques [89]. Another recent study found that variability between pulmonary lesions in the same animal was based both on the regional expression of the host response as well as anatomical

differences in microbial growth. Individual animals sterilized some lesions while others progressed in the same lung [90]. Recently, a neonatal Rhesus macaque model was found to recapitulate human TB in newborns and infants [91]. This model and others will obviously be very important in assessing novel preventative and therapeutic approaches to the "perfect storm" of HIV/TB co-infection [92, 93].

3.4.4 Rabbit

The rabbit has contributed much to our understanding of the pathogenesis of pulmonary TB for nearly a century [23, 94–98]. However, much of the early work was conducted in rabbits infected with virulent *M. bovis*, as opposed to *M. tuberculosis*. For reasons that have never been explained, rabbits are exquisitely susceptible to the former, but quite resistant to the latter [95]. Nearly all of the beautiful work on the development and fate of pulmonary lesions, including liquefaction and cavitation, was conducted with the bovine organism. Although *M. bovis* and *M. tuberculosis* share significant homology at the genetic level, and the diseases appear to share many features at the gross and histopathological levels, there has been some reluctance to fully embrace the rabbit data because it may reflect a very unique host–pathogen interaction that is not an accurate representation of human TB. On the other hand, several recent publications demonstrate that rabbits infected with high-virulence strains of *M. tuberculosis*, such as the CDC1551 and HN878 strains, provide an excellent model of chronic pulmonary TB [99, 100].

Given the major contribution of cavitary TB to transmission in humans, the ability of the rabbit to develop cavities reproducibly following aerosol exposure to virulent *M. bovis* or *M. tuberculosis* has been viewed as one of the most important contributions of this model to understanding disease pathogenesis [23, 99, 101]. In an effort to increase the frequency and predictability of cavity formation, investigators have pre-sensitized rabbits to large doses of killed *M. bovis* prior to pulmonary challenge with virulent *M. tuberculosis*. This group reported that PET-CT analysis of rabbit lungs was able to predict cavity development with good sensitivity and specificity [102]. Another group has used a skin model of BCG infection in rabbits to study liquefaction and ulceration as accessible representations of events in the lungs [103]. Highly relevant clinical outcomes associated with TB, such as latency, reactivation, and immune reconstitution inflammatory syndrome, have been modeled in the rabbit [104, 105]. Tuberculous meningitis is a serious form of extrapulmonary TB that occurs in children and has been modeled successfully in rabbits injected intrathecally with virulent strains of *M. tuberculosis* [106]. Using this model, investigators have examined the effect of antibiotic therapies [107] and vaccination in the rabbit TB meningitis model [108, 109].

Owing to cost and ABSL-3 containment requirements, rabbits have not been used for routine evaluation of novel TB vaccines or drugs [108]. However, in recent years, the size of the rabbit (a disadvantage from the biohazard housing standpoint) has been turned to an advantage for the application of cutting-edge imaging procedures to investigate the effect of drug therapies on pulmonary granulomas. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MALDI-MSI) has been used successfully to map the location of a TB drug, moxifloxacin, in rabbit lung biopsies [110, 111]. PET-CT analysis of the lungs of rabbits infected by aerosol with a high-virulence isolate (HN878) revealed that individual lesions within the same animal had very different fates,

recapitulating the heterogeneity discussed earlier. The analysis was able to detect to a treatment effect of either isoniazid or rifampin within a week of initiating therapy [112]. Yet another group utilized the rabbit TB model to investigate the effect of altered granuloma vasculature as an impediment to drug penetration into active lesions. Treatment of infected rabbits with anti-vascular endothelial growth factor (VEGF) antibody resulted in decreased granuloma hypoxia and enhanced small molecule delivery by promoting vascular normalization [113].

3.4.5 Zebrafish

The zebrafish infected with *M. marinum* has emerged as a novel model of human TB [24, 114]. Zebrafish offer several logistical advantages, including small size, ability to house large numbers in a small space, ease of breeding, and a large genetic toolbox. The fish have well-developed innate and adaptive immune systems [24]. The embryonic and larval zebrafish are transparent, a feature which facilitates direct imaging of cellular events, including granuloma formation, within the infected fish using a variety of genetic systems which employ fluorescence as a readout [115, 116]. Using this model, a number of important observations have been made which contribute to our understanding of the pathogenesis of mycobacterial infection [24, 114, 117].

Zebrafish have been used to screen anti-mycobacterial therapies [114, 118, 119]. By necessity, the drugs have been tested against *M. marinum* or *M. abscessus* since *M. tuberculosis* does not infect fish [120, 121]. This obvious difference begs the question of the relevance of drug screening in the zebrafish to predict the efficacy of drugs in mammalian TB. Indeed, a recent publication directly compared the treatment of zebrafish (infected with *M. marinum*) and mice (infected with *M. tuberculosis*) with a number of experimental compounds. The results demonstrated that some of the compounds were effective in both models; however, there were several which were efficacious in the mouse model but *not* in the zebrafish model [114]. Thus, although the zebrafish may offer a "high-throughput" system in which to test new TB drugs, the discrepancies revealed in this comparative study suggest that care should be taken in the interpretation of those screens.

Zebrafish have also been employed in the evaluation of TB vaccines. Evidence that vaccination against mycobacterial infection could actually be induced in zebrafish came from a study in which fish infected with an attenuated strain of *M. marinum* were protected against subsequent challenge with a virulent strain [122]. In a recent study, BCG and a DNA vaccine composed of genes encoding antigens that had been shown to induce protective immunity against *M. tuberculosis* in mammals were tested in zebrafish challenged with *M. marinum*. Vaccination was shown to reduce the number of granulomas, bacterial loads, and mortality [123]. The paucity of literature on vaccination in the zebrafish model does not allow any conclusion to be drawn about the value of this model in TB vaccine screening.

3.4.6 Rat

On the basis of experiments conducted more than 50 years ago, the rat was discounted as a useful TB model due to the observation that rats were extremely resistant to infection with *M. tuberculosis* [124, 125]. Indeed, high-dose respiratory infection results in the establishment of relatively stable mycobacterial populations in the lungs with little overt

evidence of disease [126]. TB granulomas in the rat have been demonstrated to be hypoxic, a condition that mimics human granulomas and may be important for inducing metabolic changes in the tubercle bacilli [127]. The need to screen large numbers of novel anti-TB drugs in recent years has stimulated a re-evaluation of the rat TB model [126, 128]. Rats are the species of choice for studies of absorption, distribution, metabolism, excretion and toxicology in early drug development and may offer an attractive alternative to mice and guinea pigs for screening novel TB drugs [129, 130]. Following endotracheal instillation of virulent mycobacteria into rats, the *ex vivo* activation of T cells by a major mycobacterial antigen, ESAT-6, was found to be a good early indicator of response to treatment with oral anti-TB drugs [131]. A recent study of standard anti-TB therapies (rifampicin, isoniazid, and ethambutol) in Wistar rats infected by the pulmonary route with virulent *M. tuberculosis* demonstrated good therapeutic efficacies following oral dosing [126]. However, given the paucity of published work on rat TB compared with other animal models, much additional work will be required to determine whether and how this model might contribute to pre-clinical TB drug evaluation.

3.4.7 Domestic Animals and Wildlife Reservoirs

As mentioned above, domestic animals (e.g., cattle, farmed deer, etc.) and wildlife reservoirs (e.g., brushtail possums in New Zealand, badgers in the UK) are naturally infected with *M. bovis* and provide more "natural" models in which to study disease pathogenesis and vaccination [28]. A comprehensive recent review makes a compelling case for the relevance and crossover value of bovine TB research for the development of tools to control human TB [132]. The ability to study novel diagnostic tests and vaccines in a host infected with its homologous pathogen has obvious advantages over the artificial pairing of laboratory-raised small animals with a pathogen that they would never encounter in the wild. In addition, vaccine trials in domestic and wild animal reservoirs of bovine TB can be designed to include natural animal-to-animal challenge, thus recapitulating the conditions of a human Phase IIb or III clinical trial [133]. Indeed, in the context of this book, novel formulations and delivery systems have been developed recently for vaccination against bovine TB, some of which may have application in human TB vaccines [134].

3.5 Summary

This book focuses on delivery systems for TB drugs and vaccines. In that context, the animal models employed in the development and pre-clinical testing of those interventions will be selected by balancing the biological relevance of the disease process in each model with the practical considerations that attend the screening of large numbers of novel biologics. As the complexities of the pathogenesis of pulmonary TB become more apparent, the "one size fits all" approach to selecting an animal model must be abandoned in favor of selecting those that faithfully mimic the precise disease features against which the drug or vaccine is intended to work. This is especially important in testing drugs which must act on diverse bacterial populations within the same host that vary in their replicative and metabolic potential. Each animal model offers unique advantages that might make it the model of choice in a particular research application. Recent improvements in the major models

include the application of cutting-edge imaging technologies (rabbit, NHP), the development of immunological reagents (guinea pig), and the use of inbred mouse strains that produce hypoxic, necrotic TB granulomas. As the subsequent chapters will illustrate, these models have been put to good use in the search for the next generation of TB drugs and vaccines.

References

[1] Wiegeshaus, E.E. and Smith, D.W. (1989) Evaluation of the protective potency of new tuberculosis vaccines, *Rev. Infect. Dis.*, **11**, S484–S490.

[2] Smith, D.W., Grover, A.A. and Wiegeshaus, E.H. (1968) Nonliving immunogenic substances of mycobacteria, *Adv. Tuberc. Res.*, **16**, 191–227.

[3] Ly, L.H. and McMurray, D.N. (2008) Tuberculosis: vaccines in the pipeline. *Exp. Rev. Vacc.*, **7**, 635–650.

[4] Wiegeshaus, E.H., Harding, G., McMurray, D. *et al*. (1971) A cooperative evaluation of test systems used to assay tuberculosis vaccines, *Bull. WHO*, **45**, 543–550.

[5] Smith, D.W., Wiegeshaus, E.H., Stark, R.H. and Harding, G.E. (1972) Models for potency assay of tuberculosis vaccines, *Fogarty Intl. Ctr. Proc.*, **14**, 205–218.

[6] Izzo, A., Brandt, L., Lasco, T. *et al*. (2005) NIH pre-clinical screening program: overview and current status, *Tuberculosis*, **85**, 25–28.

[7] Williams, A., Hatch, G.J., Clark, S.O. *et al*. (2005) Evaluation of vaccines in the EU TB Vaccine Cluster using a guinea pig aerosol infection model of tuberculosis, *Tuberculosis*, **85**, 29–38.

[8] Grover, A., Troudt, J., Arnett, K. *et al*. (2012) Assessment of vaccine testing at three laboratories using the guinea pig model of tuberculosis, *Tuberculosis*, **92**, 105–111.

[9] Young, D. (2009) Infectious disease: Tuberculosis, *Eur. J. Immunol.*, **39**, 2011–2014.

[10] Dey, B. and Bishai, W.R. (2014) Crosstalk between *Mycobacterium tuberculosis* and the host cell, *Sem. Immunol.*, **26**, 486–496.

[11] Warner, D.F., Koch, A. and Mizrahi, V. (2015) Diversity and disease pathogenesis in *Mycobacterium tuberculosis, Trends Microbiol*., **23**, 14–21.

[12] Bumann, D. (2015) Heterogeneous host-pathogen encounters: act locally, think globally, *Cell Host Microbe*, **17**, 13–19.

[13] Orme, I.M. and Basaraba, R.J. (2014) The formation of the granuloma in tuberculosis infection, *Sem. Immunol.*, **26**, 601–609.

[14] Behar, S.M., Carpenter, S.M., Booty, M.G. *et al*. (2014) Orchestration of pulmonary T cell immunity during *Mycobacterium tuberculosis* infection: immunity interruptus, *Sem. Immunol.*, **26**, 559–577.

[15] Nunes-Alves, C., Booty, M.G., Carpenter, S.M. *et al*. (2014) In search of a new paradigm for protective immunity to TB, *Nat. Rev. Microbiol.*, **12**, 289–299.

[16] Jain, S.K., Hernandez-Abanto, S.M., Cheng, Q.-J. *et al*. (2007) Accelerated detection of *Mycobacterium tuberculosis* genes essential for bacterial survival in guinea pigs compared with mice, *J. Infect. Dis.*, **195**, 1634–1642.

[17] Gillespie, S.H., Crook, A.M., McHugh, T.D. *et al*. (2014) for the REMox TB Consortium, Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis, *N. Engl. J. Med.*, **371**, 1577–1587.

[18] Tameris, M.D., Hatherill, M., Landry, B.S. *et al*. (2014) Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomized, placebo-controlled phase 2b trial, *Lancet*, **381**, 1021–1028.

[19] McShane, H. and Williams, A. (2014) A review of preclinical animal models utilized for TB vaccine evaluation in the context of recent human efficacy data, *Tuberculosis*, **94**, 105–110.

[20] Clark, S., Hall, Y. and Williams, A. (2015) Animal models of tuberculosis: guinea pigs, *Cold Spring Harbor Perspect. Med.*, **5**, a018572.

[21] Cooper, A.M. (2015) Mouse model of tuberculosis, *Cold Spring Harbor Perspect. Med.*, **5**, a018556.

[22] Scanga, C.A. and Flynn, J.L. (2014) Modeling tuberculosis in nonhuman primates, *Cold Spring Harbor Perspect. Med.*, **4**, a018564.
[23] Helke, K.L., Mankowski, J.L. and Manabe, Y.C. (2005) Animal models of cavitation in pulmonary tuberculosis, *Tuberculosis*, **86**, 337–348.
[24] van Leeuwen, L.M., van der Sar, A.M. and Bitter, W. (2015) Animal models of tuberculosis: zebrafish, *Cold Spring Harbor Perspect. Med.*, **5**, a018580.
[25] Barker, L., Hessel, L. and Walker, B. (2012) Rational approach to selection and clinical development of TB vaccine candidates, *Tuberculosis*, **92S1**, S25–S29.
[26] Brennan, M.J. and Thole, J. (2012) Tuberculosis vaccines: a strategic blueprint for the next decade, *Tuberculosis*, **92S1**, S6–S13.
[27] Principi, N. and Esposito, S. (2015) The present and future of tuberculosis vaccinations, *Tuberculosis*, **95**, 6–13.
[28] Young, D. and Verreck, F.A.W. (2012) Creativity in tuberculosis research and discovery, *Tuberculosis*, **92S1**, S14–S16.
[29] Lenaerts, A.J., DeGroote, M.A. and Orme, I.M. (2007) Preclinical testing of new drugs for tuberculosis: current challenges, *Trends Microbiol.*, **16**, 48–54.
[30] Nuesrmberger, E. (2008) Using animal models to develop new treatments for tubercuosis, *Semin. Respir. Crit. Care Med.*, **29**, 542–551.
[31] Basaraba, R.J. (2008) Experimental tuberculosis: the role of comparative pathology in the discovery of improved tuberculosis treatment strategies, *Tuberculosis*, **88**, S35-S47.
[32] Franzblau, S.G., DeGroote, M.A., Cho, S.H. *et al*. (2012) Comprehensive analysis of methods used for the evaluation of compounds against *Mycobacterium tuberculosis, Tuberculosis*, **92**, 453–488.
[33] Dartois, V. (2014) The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells, *Nat. Rev. Microbiol.*, **12**, 159–167.
[34] Mdluli, K., Kaneko, T. and Upton, A. (2015) The tuberculosis drug discovery and development pipeline and emerging drug targets, *Cold Spring Harbor Perspect. Med.*, **5**, a021154.
[35] McMurray, D.N. (1994) Guinea pig model of tuberculosis, In *Tuberculosis: Pathogenesis, Protection and Control* (ed. B.R. Bloom), American Society for Microbiology, Washington, D.C., pp. 135–147.
[36] Padilla-Carlin, D.J., McMurray, D.N. and Hickey, A.J. (2008) The guinea pig as a model of infectious diseases, *Comp. Med.*, **58**, 324–340.
[37] McMurray, D.N. (2001) Disease model: pulmonary tuberculosis, *Trends Mol. Med.*, **7**, 135–137.
[38] Hoff, D.R., Ryan, G.J., Driver, E.R *et al*. (2011) Location of intra- and extracellular *M. tuberculosis* populations in lungs of mice and guinea pigs during disease progression and after drug treatment, *PLoS One*, **6**, e17550.
[39] Ryan, G.J., Hoff, D.R., Driver, E.R *et al*. (2010) Multiple *M. tuberculosis* phenotypes in mouse and guinea pig lung tissue revealed by a dual-staining approach, *PLoS One*, **5**, e11108.
[40] Turner, O.C., Basaraba, R.J. and Orme, I.M. (2003) Immunopathogenesis of pulmonary granulomas in the guinea pig after infection with *Mycobacterium tuberculosis*, *Infect. Immun.*, **71**, 864–871.
[41] McMurray, D.N. (2003) Heterogeneous reseeding of the lung in low-dose, aerosol-infected guinea pigs: unique features of the host-pathogen interface in secondary tubercles, *Tuberculosis*, **83**, 131–134.
[42] Via, L.E., Lin, P.L., Ray, S.M. *et al*. (2006) Tuberculosis granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates, *Infect. Immun.*, **76**, 2333–2340.
[43] Dutta, N.K., Illei, P.B., Peloquin, P.A. *et al*. (2012) Rifapentine is not more active than rifampin against chronic tuberculosis in guinea pigs, *Antimicrob. Agents Chemother.*, **56**, 3726–3731.
[44] Dutta, N.K., Alsultan, A., Gniadek, T.J. *et al*. (2013) Potent rifamycin-sparing regimen cures guinea pig tuberculosis as rapidly as the standard regimen, *Antimicrob. Agents Chemother.*, **57**, 3910–3916.
[45] Lenaerts, A.J., Hoff, D., Aly, S. *et al*. (2007) Location of persisting mycobacteria in a guinea pig model of tuberculosis revealed by r207910, *Antimicrob. Agents Chemother.*, **51**, 3338–3345.

[46] Schafer, H. and Burger, R. (2012) Tools for cellular immunology and vaccine research in the guinea pig: monoclonal antibodies to cell surface antigens and cell lines, *Vaccine*, **30**, 5804–5811.
[47] Ordway, D., Palanisamy, G., Henao-Tamayo, M. *et al*. (2007) The cellular immune response to *Mycobacterium tuberculosis* infection in the guinea pig, *J. Immunol.*, **179**, 2532–2541.
[48] Ordway, D., Henao-Tamayo, H., Shanley, C. *et al*. (2008) Influence of *Mycobacterium bovis* BCG vaccination on cellular immune response of guinea pigs challenged with *Mycobacterium tuberculosis*, *Clin. Vaccine Immunol.*, **15**, 1248–1258.
[49] Campbell, E.M., Proudfoot, A.E., Yoshimura, T. *et al*. (1997) Recombinant guinea pig and human RANTES activate macrophages but not eosinophils in the guinea pig, *J. Immunol.*, **159**, 1482–1489.
[50] White, A.M., Yoshimura, T., Smith, A.W. *et al*. (1997) Airway inflammation induced by recombinant guinea pig tumor necrosis factor-alpha, *Am. J. Physiol.*, **273**, L524–530.
[51] Yoshimura, T. (1993) cDNA cloning of guinea pig monocyte chemoattractant protein-1 and expression of the recombinant protein, *J. Immunol.*, **150**, 5025–5032.
[52] Yoshimura, T. and Johnson, D.G. (1993) cDNA cloning and expression of guinea pig neutrophil attractant protein-1 (NAP-1): NAP-1 is highly conserved in guinea pig, *J. Immunol.*, **151**, 6225–6236.
[53] McMurray, D.N., Allen, S.S., Jeevan, A. *et al*. (2005) Vaccine-induced cytokine responses in a guinea pig model of pulmonary tuberculosis, *Tuberulosis.*, **85**, 295–301.
[54] Jeevan, A., McFarland, C.T., Yoshimura, T. *et al*. (2006) Production and characterization of guinea pig recombinant interferon-gamma: effect on macrophage activation, *Infect. Immun.*, **74**, 213–224.
[55] Jeevan, A., Yoshimura, T., Ly, L.H. *et al*. (2011) Cloning of guinea pig IL-4: reduced IL-4 mRNA after vaccination or *Mycobacterium tuberculosis* infection, *Tuberculosis*, **91**, 47–56.
[56] Dirisala, V.R., Jeevan, A., Bix, G. *et al*. (2012) Molecular cloning and expression of the IL-10 gene from guinea pigs, *Gene*, **498**, 120–127.
[57] Dirisala, V.R., Jeevan, A., Ly, L.H. and McMurray, D.N. (2013) Prokaryotic expression and in vitro functional analysis of IL-1β and MCP-1 from Guinea pig, *Mol. Biotechnol.*, **54**, 312–319.
[58] Dirisala, V.R., Jeevan, A., Ramasamy, S.K. and McMurray, D.N. (2013) Molecular cloning, expression and in silico structural analysis of guinea pig IL-17, *Mol. Biotechnol.*, **55**, 277–287.
[59] Cho, H. and McMurray, D.N. (2005) Neutralization of TNFα suppresses antigen-specific Type 1 cytokine responses and reverses the inhibition of mycobacterial survival in cocultures of immune guinea pig T lymphocytes and infected macrophages, *Infect. Immun.*, **73**, 8437–8441.
[60] Cho, H. and McMurray, D.N. (2007) Recombinant guinea pig TNFα enhances antigen-specific type 1 T lymphocyte activation in guinea pig splenocytes, *Tuberculosis*, **87**, 87–93.
[61] Kramp, J.C., McMurray, D.N., Formichella, C. and Jeevan, A. (2011) Immunomodulatory effect of recombinant tumor necrosis factor-alpha in guinea pigs vaccinated with *Mycobacterium bovis* bacille Calmette-Guerin, *Clin. Exp. Immunol.*, **165**, 110–120.
[62] Allen, S.S., Cassone, L., Lasco, T.M. *et al*. (2004) Effect of neutralizing transforming growth factor-beta1 on the immune response against *Mycobacterium tuberculosis* in guinea pigs, *Infect. Immun.*, **72**, 1358–1363.
[63] Ly, L.H., Jeevan, A. and McMurray, D.N. (2009) Neutralization of TNFalpha alters inflammation in guinea pig tuberculous pleuritis, *Microbes Infect.*, **11**, 680–688.
[64] Allen, S.S., Mackie, J.T., Russell, K. *et al*., (2008) Altered inflammatory responses following transforming growth factor-beta neutralization in experimental guinea pig tuberculous pleuritis, *Tuberculosis*, **88**, 430–436.
[65] Ly, L.H., Russell, M.I. and McMurray, D.N. (2007) Microdissection of the cytokine milieu of pulmonary granulomas from tuberculous guinea pigs, *Cell. Microbiol.*, **9**, 1127–1136.
[66] Ly, L.H., Russell, M.I. and McMurray, D.N. (2008) Cytokine profiles in primary and secondary pulmonary granulomas of guinea pigs with tuberculosis, *Am. J. Resp. Cell Mol. Biol.*, **38**, 455–462.
[67] Nikonenko, B.V. and Apt, A.S (2013) Drug testing in mouse models of tuberculosis and nontuberculous mycobacterial infections, *Tuberculosis*, **93**, 285–290.

[68] Morello, M., Krone, C.L., Dickerson, S. *et al*. (2009) Dry-powder pulmonary insufflation in the mouse for application to vaccine and drug studies, *Tuberculosis*, **89**, 371–377.
[69] DeGroote, M.A., Gruppo, V., Woolhiser, L.K. *et al*. (2012) Importance of confirming data on the *in vivo* efficacy of novel antibacterial drug regimens against various strains of *Mycobacterium tuberculosis*, *Antimicrob. Agents Chemother.*, **56**, 731–738.
[70] Rosenthal, I.M., Tasneen, R., Peloquin, C.A. *et al*. (2012) Dose-ranging comparison of rifampin and rifapentine in two pathologically distinct murine models of tuberculosis, *Antimicrob. Agents Chemother.*, **56**, 4331–4340.
[71] DeGroote, M.A., Gilliland, J.C., Wells, C.L. *et al*. (2011) Comparative studies evaluating mouse models used for efficacy testing of experimental drugs against *Mycobacterium tuberculosis*, *Antimicrob. Agents Chemother.*, **55**, 1237–1247.
[72] Kramnik, I., Demant, P. and Bloom, B.R. (1998) Susceptibility to tuberculosis as a complex genetic trait: analysis using recombinant congenic strains of mice, *Novartis Found. Symp.*, **217**, 120–132.
[73] Kramnik, I., Dietrich, W.F., Demant, P. and Bloom, B.R. (2000) Genetic control of resistance to experimental infection with virulent *Mycobacterium tuberculosis*, *Proc. Natl. Acad. Sci. USA*, **97**, 8560–8565.
[74] Harper, J., Skerry, C., Davis, S.L *et al*. (2012) Mouse model of necrotic tuberculosis granulomas develops hypoxic lesions, *J. Infect. Dis.*, **205**, 595–602.
[75] Driver, E.R., Ryan, G.J., Hoff, D.R. *et al*. (2012) Evaluation of a mouse model of necrotic granuloma formation using C3HeB/FeJ mice for testing of drugs against *Mycobacterium tuberculosis*, *Antimicrob. Agents Chemother.*, **56**, 3181–3195.
[76] Kager, L.M., Runge, J.H., Nederveen, A.J. et al. (2014) A new murine model to study musculoskeletal tuberculosis (short communication), *Tuberculosis*, **94**, 306–310.
[77] de Steenwinkel, J.E.M., de Knegt, G.J., den Kate, M.T. *et al*. (2013) Relapse of tuberculosis versus primary tuberculosis: course, pathogenesis and therapy in mice, *Tuberculosis*, **93**, 213–221.
[78] Leung, C., Chijioke, O., Gujer, C. *et al*. (2013) Infectious diseases in humanized mice, *Eur. J. Immunol.*, **43**, 2246–2254.
[79] Calderon, V.E., Valbuena, G., Goez, Y. *et al*. (2013) A humanized mouse model of tuberculosis, *PLoS One*, **8**, e63331.
[80] Heutz, F., Gavier-Widen, D., Carow, B. *et al*. (2013) CD4+ cell-dependent granuloma formation in humanized mice infected with mycobacteria, *Proc. Natl. Acad. Sci. USA*, **110**, 6482–6487.
[81] Manina, G., Dhar, N. and McKinney, J.D. (2015) Stress and host immunity amplify *Mycobacterium tuberculosis* phenotypic heterogeneity and induce nongrowing metabolically active forms, *Cell Host Microbe*, **17**, 32–46.
[82] Sukumar, N., Tan, S., Aldridge, B.B. and Russell, D.G. (2014) Exploitation of *Mycobacterium tuberculosis* reporter strains to probe the impact of vaccination at sites of infection, *PLoS Pathogens*, **10**, e1004394.
[83] McMurray, D.N. (2000) A nonhuman primate model for preclinical testing of new tuberculosis vaccines, *Clin. Infect. Dis.*, **30(Suppl 3)**, S210–212.
[84] Walsh, G.P., Tan, E.V., de la Cruz, E.C. *et al*. (1996) The Philippine cynomolgus monkey (*Macaca fascicularis*) provides a new nonhuman primate model of tuberculosis that resembles human disease, *Nat. Med.*, **2**, 430–436.
[85] Langermans, J.A.M., Andersen, P., van Soolingen, D. *et al*. (2001) Divergent effect of bacillus Calmette-Guerin (BCG) vaccine in highly related macaque species: implications for primate models in tuberculosis vaccine research, *Proc. Natl. Acad. Sci. USA*, **98**, 11497–11502.
[86] Pena, J.C. and Ho, W.-Z. (2015) Monkey models of tuberculosis: lessons learned, *Infect. Immun.*, **83**, 852–862.
[87] Via, L.E., Weiner, D.M., Schimel, D. *et al*. (2013) Different virulence and disease progression following *Mycobacterium tuberculosis* complex infection of the common marmoset (*Callithrix jacchus*), *Infect. Immun.* **81**, 2909–2919.

[88] Sharpe, S.A., McShane, H., Dennis, M.J. *et al*. (2010) Establishment of an aerosol challenge model of tuberculosis in Rhesus macaques and an evaluation of endpoints for vaccine testing, *Clin. Vaccine Immunol.*, **17**, 1170–1182.
[89] Lin, P.L., Coleman, T., Carney, J.P.J. *et al*. (2013) Radiologic responses in cynomolgus macaques for assisting tuberculosis chemotherapy regimens, *Antimicrob. Agents Chemother.*, **57**, 4237–4244.
[90] Lin, P.L., Ford, C.B., Coleman, M.T. *et al*. (2014) Sterilization of granulomas is common in active and latent tuberculosis despite within-host variability in bacterial killing, *Nat. Med.*, **20**, 75–79.
[91] Cepeda, M., Salas, M., Folwarczny, J. *et al*. (2013) Establishment of a neonatal rhesus macaque model to study *Mycobacterium tuberculosis infection*, *Tuberculosis*, **93S1**, S51–S59.
[92] Jensen, K., Ranganathan, U.D., van Rompay, K.K. *et al*. (2012) A recombinant attenuated *Mycobacterium tuberculosis* vaccine strain is safe in immunosuppressed simian immunodeficiency virus-infected infant macaques, *Clin. Vaccine Immunol*.,**19**, 1170–1181.
[93] Diedrich, C.R., Mattila, J.T. and Flynn, J.L. (2013) Monocyte-derived IL-5 reduces TNF production by *Mycobacterium tuberculosis*-specific CD4 T cells during SIV/*M. tuberculosis* co-infection, *J. Immunol.*, **190**, 6320–6328.
[94] Converse, P.J., Dannenberg, A.M., Shigenaga, T. *et al*. (1998) Pulmonary bovine-type tuberculosis in rabbits: bacillary virulence, inhaled dose effects, tuberculin sensitivity, and *Mycobacterium vaccae* immunotherapy, *Clin. Diagn. Lab. Immunol.*, **5**, 871–881.
[95] Lurie, M.B. (1928) The fate of human and bovine tubercle bacilli in various organs of the rabbit, *J. Exp. Med.*, **48**, 155–182.
[96] Lurie, M.B., Abramson, S. and Heppleston, A.G. (1952) On the response of genetically resistant and susceptible rabbits to the quantitative inhalation of human-type tubercle bacilli and the nature of resistance to tuberculosis, *J. Exp. Med.*, **95**, 119–134.
[97] Ratcliffe, H.L. and Wells, W.F. (1948) Tuberculosis of rabbits induced by droplet nuclei infection, *J. Exp. Med.*, **87**, 575–584.
[98] Dannenberg, A.M. (2006) *Pathogenesis of human pulmonary tuberculosis: insights from the rabbit model*, ASM Press, Washington DC.
[99] Subbian, S., Tsenova, L., Yang, G. *et al*. (2011) Chronic pulmonary cavitary tuberculosis in rabbits: a failed host immune response, *Open Biol.*, **1**, e110016.
[100] Subbian, S., Bandyopadhyay, N., Tsenova, L. *et al*. (2013) Early innate immunity determines outcome of *Mycobacterium tuberculosis* pulmonary infection in rabbits, *Cell Commun. Signal.*, **11**, 60–77.
[101] Converse, P.J.,,Dannenberg, A.M., Estep, J. *et al*. (966) Cavitary tuberculosis produced in rabbits by aerosolized virulent tubercle bacilli, *Infect. Immun.*, **64**, 4776–4787.
[102] Luna, B., Kubler, A., Larsson, C. *et al*. (2015) In vivo prediction of tuberculosis-associated cavity formation in rabbits, *J. Infect. Dis.*, **211**, 481–485.
[103] Sun, H., Ma, X., Zhang, G. *et al*. (2012) Effects of immunomodulators on liquefaction and ulceration in the rabbit skin model of tuberculosis, *Tuberculosis*, **92**, 345–350.
[104] Manabe, Y.C., Kesavan, A.K., Lopez-Molina, J. *et al*. (2008) The aerosol rabbit model of TB latency, reactivation and immune reconstitution inflammatory syndrome, *Tuberculosis*, **88**, 187–196.
[105] Subbian, S., O'Brien, P., Kushner, N.L. *et al*. (2013) Molecular immunologic correlates of spontaneous latency in a rabbit model of pulmonary tuberculosis, *Cell Commun. Signal.*, **11**, 16–32.
[106] Tsenova, L., Ellison, E., Harbacheuski, R. *et al*. (2005) Virulence of selected *Mycobacterium tuberculosis* clinical isolates in the rabbit model of meningitis is dependent on phenolic glycolipid produced by the bacilli, *J. Infect. Dis.*, **192**, 98–106.
[107] Tsenova, L., Sokol, K., Freedman, V.H. and Kaplan, G.A. (1998) A combination of thalidomide plus antibiotics protects rabbits from mycobacterial meningitis-associated deaths, *J. Infect. Dis.*, **177**, 1563–1572.
[108] Tsenova, L., Harbacheuski, R., Sung, N. *et al*. (2007) BCG vaccination confers poor protection against *M. tuberculosis* HN878-induced central nervous system disease, *Vaccine*, **25**, 5126–5132.

[109] Tsenova, L., Harbacheuski, R., Moreira, A.L. *et al*. (2006) Evaluation of the Mtb72F polyprotein vaccine in a rabbit model of tuberculous meningitis, *Infect. Immun.*, **74**, 2392–2401.
[110] Prideaux, B., Dartois, V., Staab, D. *et al*. (2011) High-sensitivity MALDI-MRM-MS imaging of moxifloxacin distribution in tuberculosis-infected rabbit lungs and granulomatous lesions, *Anal. Chem.*, **83**, 2112–2118.
[111] Bagci, U., Foster, B., Miller-Jaster, K. *et al*. (2013) A computational pipeline for quantification of pulmonary infections in small animal models using serial PET-CT imaging, *EJNMMI Res.*, **3**, 55-75.
[112] Via, L.E., Schimel, D., Weiner, D.M. *et al*. (2012) Infection dynamics and response to chemotherapy in a rabbit model of tuberculosis using [^{18}F] 2-fluoro-deoxy-D-glucose positron emission tomography and computed tomography, *Antimicrob. Agents Chemother.*, **56**, 4391–4402.
[113] Datta, M., Via, L.E., Kamoun, W.S. *et al*. (2015) Anti-vascular endothelial growth factor treatment normalizes tuberculosis granuloma vasculature and improves small molecule delivery, *Proc. Natl. Acad. Sci. USA*, **112**, 1827–1832.
[114] Berg, R.D. and Ramakrishnan, L. (2012) Insights into tuberculosis from the zebrafish model, *Trends Mol. Med.*, **18**, 689–690.
[115] Ramakrishnan, L. (2013) Looking within the zebrafish to understand the tuberculous granuloma, *Adv. Exp. Med. Biol.*, **783**, 251–266.
[116] Tobin, D.M., May, R.C. and Wheeler, R.T (2012) Zebrafish: a see-through host and a fluorescent toolbox to probe host-pathogen interaction, *PLoS Pathog.* **8**, e1002349.
[117] Parikka, M., Hammaren, M.M., Harjula, S.K. *et al*. (2012) *Mycobacterium marinum* causes a latent infection that can be reactivated by γ irradiation in adult zebrafish, *PLoS Pathog.*, **8**, e1002944.
[118] Ordas, A., Raterink, R.-J., Cunningham, F. *et al*. (2015) Testing tuberculosis drug efficacy in a zebrafish high-throughput translational medicine screen, *Antimicrob. Agents Chemother.*, **59**, 753–762.
[119] Adams, K.N., Takaki, K., Connolly, L.E. *et al*. (2011) Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism, *Cell*, **145**, 39–53.
[120] Bernut, A., Le Moigne, V., Lesne, T. *et al*. (2014) In vivo assessment of drug efficacy against *Mycobacterium abscessus* using the embryonic zebrafish test system, *Antimicrob. Agents Chemother.*, **58**, 4054–4063.
[121] Takaki, K., Cosma, C.L., Troll, M.A. and Ramakrishnan, L. (2012) An in vivo platform for rapid high-throughput antitubercular drug discovery, *Cell Rep.*, **2**, 175–184.
[122] Cui, Z., Samuel-Shaker, D., Watral, V. and Kent, M.L. (2010) Attenuated *Mycobacterium marinum* protects zebrafish against mycobacteriosis, *J. Fish Dis.*, **33**, 371–375.
[123] Oksanen, K.E., Halfpenny, N.J.A., Sherwood, E. *et al*. (2013) An adult zebrafish model for preclinical tuberculosis vaccine development, *Vaccine*, **31**, 5202–5209.
[124] Ornstein, G.B. and Steinbach, M.M. (1925) The resistance of the albino rat to infection with tubercle bacilli, *Am. Rev. Tuberc.*, **12**, 77–86.
[125] Wessels, C.C. (1941) Tuberculosis in the rat. II. The fate of tubercle bacilli in the various organs of the rat, *Am. Rev. Tuberc.*, **43**, 459–474.
[126] Kumar, N., Vishwas, K.G., Kumar, M. *et al*. (2014) Pharmacokinetics and dose response of anti-TB drugs in rat infection model of tuberculosis, *Tuberculosis*, **94**, 282–286.
[127] Heng, Y., Seah, P.G., Siew, J.Y. *et al*. (2011) *Mycobacterium tuberculosis* infection induces hypoxic lung lesions in the rat, *Tuberculosis*, **91**, 339–341.
[128] Sugawara, I., Yamada, H. and Mizuno, S. (2004) Pathological and immunological profiles of rat tuberculosis, *Int. J. Exp. Pathol.*, **85**, 125–134.
[129] Singhal, A., Aliouat, E.M., Herve, M. et al. (2011) Experimental tuberculosis in the Wistar rat: a model for protective immunity and control of infection, *PLoS One*, **6**, e18632.
[130] Baldan, H.M., De Rosa, H.J., Brunetti, I.L. et al. (2007) The effect of rifampicin and pyrazinamide on isoniazid pharmacokinetics in rats, *Biopharm. Drug Dispos.*, **28**, 409–413.

[131] Foo, D.G., Tay, H.C., Siew, Y. *et al*. (2011) T cell monitoring of chemotherapy in experimental rat tuberculosis, *Antimicrob. Agents Chemother.*, **55**, 3677–3683.
[132] Waters, W.R., Maggioli, M.F., McGill, J.L. *et al*. (2014) Relevance of bovine tuberculosis research to the understanding of human disease: historical perspectives, approaches, and immunologic mechanisms, *Vet. Immunol. Immunopathol.*, **159**, 113–132.
[133] Buddle, B.M., Parlane, N.A., Wedlock, D.N. and Heiser, A. (2013) Overview of vaccination trials for control of tuberculosis in cattle, wildlife and humans, *Transbound. Emerg. Dis.*, **60**, 136–146.
[134] Parlane, N.A., Rehm, B.H.A., Wedlock, D.N. and Buddle, B.M. (2014) Novel particulate vaccines utilizing polyester nanoparticles (bio-beads) for protection against *Mycobacterium bovis* infection – a review, *Vet. Immunol. Immunopathol.*, **158**, 8–13.

Section 2

Immunological Intervention

4

Vaccine Preparation: Past, Present, and Future

Dominique N. Price, Nitesh K. Kunda*, Amber A. McBride and Pavan Muttil*

Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico, Albuquerque, New Mexico, USA

4.1 Introduction

Vaccines are the most effective tools in preventing infectious diseases [1]. Vaccines prime the host adaptive immune system against a particular infectious agent [2]. Therefore, much attention is paid to the antigen which elicits host immunity; these are often an attenuated or weakened version of the disease-causing pathogen, although they can also be toxins or surface antigens. However, there is more to vaccine formulation than just the choice of antigen. Effectiveness can be modulated by many parameters outside of antigen selection. A successful vaccine is safe, stable, affordable, easily stored and distributed, in addition to being effective.

Within this chapter we will explore tuberculosis (TB) vaccine design, formulation, preparation, and manufacture; beginning with the first preparations of bacillus Calmette–Guérin (BCG) administered, to the current formulation, and new preparations in preclinical trials. The focus of the chapter, however, will be formulation and manufacture after the antigenic component of vaccine design. Figure 4.1 illustrates the process of vaccine preparation, beginning with formulation and ending with mass production and distribution. This is a simplified perspective of vaccine preparation, as it removes much of the administrative and bureaucratic review including clinical trials and the US Food and Drug Administration

*Authors contributed equally to this chapter

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

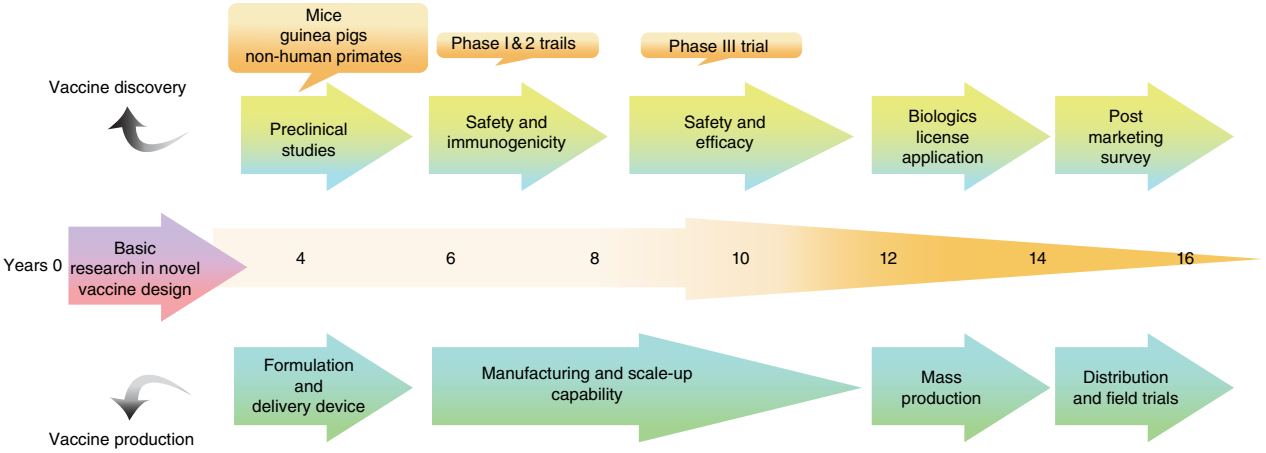


Figure 4.1 *Overview of the vaccine formulation and development process. Vaccine discovery, formulation, manufacture, and regulatory approval processes make the time to mass distribution a lengthy ordeal. Aligning the discovery with production will shorten the time from bench to bedside*

(FDA)-approval process. However, it defines the characteristics of vaccine design that must be evaluated for the initial submission of a successful candidate.

Starting with formulation, many vaccines require excipients and diluents for effective immunization. The best example of this is the addition of adjuvants to augment the generated immune response to the infectious agent. Many vaccines against other infectious pathogens use adjuvants to boost immunity to poorly immunogenic antigens [3–6]. Excipients influence the stability of the vaccine, and are chosen based on the intended route of delivery for effective administration [7]. Currently, the World Health Organization (WHO) estimates that approximately 50% of vaccines manufactured become inactive due to temperature instability [8]. This may be partially due to the fact that most vaccines are still liquid formulations, requiring refrigeration. Furthermore, injectable formulations run the risk of needle-stick injuries and generate additional costs associated with administration and training [9–11].

Manufacture incorporates the feasibility of production. Vaccines must be produced in mass quantities, while maintaining Quality Control (QC), and Good Manufacturing Practices (GMP). The manufacturing component of design is often associated with cost–benefit analysis, producing vaccines that are safe, effective and affordable. Mass production, transportation, packaging, and administration instructions affect how the vaccine will be received and used, especially in the low- and middle-income countries (LMICs) where TB is more prevalent. This chapter aims to explore areas of research and development outside of antigen choice, an area in need of innovation and novel ideas.

4.2 Early Efforts in TB Vaccine Development

The *Mycobacterium bovis* (*M. bovis)* BCG vaccine dates to the early 1900s in Lille, France when Albert Calmette and Camille Guérin cultivated tubercle bacilli on potato medium using bile and glycerin detergents to prevent the bacteria from clumping [12]. Over the next few years, tubercle bacillus was subcultured 230 times in an attempt to reduce its pathogenicity [13], leading to a weakened virulent strain. The subcultured strain was attenuated such that it did not produce progressive TB when injected into cattle, horses, guinea pigs, or rabbits, but retained much of its antigenicity [14].

4.2.1 Early BCG Formulation and Manufacturing

After 1921, the BCG culture was grown in Sauton's medium [15]. The vaccine underwent multiple processes akin to manufacturing: concentration by filtration, de-agglomeration using the physical process of ball milling, and finally, the bacteria were resuspended in protective solutions for stabilization. However, the number of colony-forming units (CFU) differed from batch to batch due to varying liquid volume. In addition, the degree of dispersion, the killing of BCG during the ball mill homogenization process, and differences in the manufacturing processes all contributed to the variability in CFU. Taken together, these variabilities highlight a lack of manufacturing regulation [16].

Neither cold-chain storage, cryopreservation, nor freeze-drying (lyophilization) technology was available in the 1920s. As a result, BCG continued to be grown and passaged in the same conditions from 1921 through 1961, until the lyophilization of BCG-Pasteur, for a total of 1173 passages [17]. Attenuated bovine tubercle bacillus has ultimately become

today's BCG vaccine. While historical and genetic records provide irrefutable evidence that BCG strains have evolved since 1921, discernible effects of genetic changes to the bacteria and their capacity to serve as an effective vaccine are still in question. The BCG vaccine is similar to *Mycobacterium tuberculosis (M. tb)* with some structural and functional differences between the two strains [18]. At this time, the full genome of BCG has been elucidated – 129 genes are absent from BCG that are present in *M. tb* [19]. There have been 49 substrains of BCG vaccine in production and in use at one time or another [20].

4.2.2 History of the BCG Vaccine and Routes of Administration

In 1921, the oral BCG vaccine was first administered to an infant [21]. Initially, the oral route was chosen for BCG administration because the gastrointestinal tract is the natural route of infection for *M. bovis*. In addition, children's intestinal mucosa have greater permeability to BCG compared to the intestinal mucosa of an adult. In 1924, the oral BCG vaccine was deemed safe and effective, prepared as an emulsion by Boquet and Negre [22]. The initial oral BCG vaccine was swallowed and then followed with either water, fruit juice, or milk. However, these diluents did not provide any protection from the harsh gastric environment [23]. The Pasteur Institute in Lille began to mass produce the oral BCG vaccine. In 1924, BCG lots were distributed to various countries for preparation using the same passage conditions as those used at the Pasteur Institute, notably lacking any GMP regulation or cold-chain management during transportation. Between 1924 and 1928, approximately 114,000 children were immunized without serious complications [24]. Later, when the intradermal and subcutaneous delivery routes were used, they caused local reactions to the vaccine [19].

The intradermal route became a popular route of delivery after 1927 when Wallgreen refined the intradermal vaccination, inoculating 0.1 mg of BCG in individuals of any age. A change in the delivery route, from oral to the parenteral, was instituted due to patients' poor immunization response to the purified protein derivative (PPD) tuberculin skin test [25]. Studies performed by scientists at the Escola Paulista de Medicina in Brazil strongly influenced the change of immunization route. Moreover, they observed that individuals immunized with oral BCG had no delayed hypersensitivity response to the cutaneous test, unlike those immunized by the intradermal route [26]. Economical and operational reasons also contributed to a change from the oral route [27]. Oral vaccination was discontinued during the 1960s, with the exception of a few countries [25].

Brazil predominantly administers BCG by the intradermal route. However, the Ataulpho de Paiva Foundation in Brazil manufactures the oral BCG Moreau strain, producing 10,000 doses per year. Their rationale is that the oral route is more immunogenic with fewer side effects compared to the parenterally administered BCG [19, 26, 28]. In addition, the oral BCG immunization requires booster doses for the first 6 months of a child's life, as mandated by National Policy of the Brazilian government [23]. The effectiveness of oral BCG immunization is widely debated.

4.2.3 Quality Control Issues

A serious BCG vaccine-manufacturing accident occurred in Lubeck, Germany in 1930 that caused a change in BCG vaccination. Of a group of 250 children vaccinated, 73 children died from TB one year after vaccination and another 135 developed signs and symptoms of the disease [24]. Investigation revealed that a culture of *M. tb* was kept in the same

incubator with the BCG. The preparation was contaminated and ultimately contained 1/3 of BCG and 2/3 of the tubercle bacillus [29]. This example highlights one of the many lessons learned. Good laboratory practice and manufacturing regulation were missing from early development, leading to variability in effectiveness. Today, QC of the current BCG vaccines used in National Immunization Programs is the responsibility of the individual manufacturers, requiring continual oversight by an independent and competent National Control Authority (NCA) in the country of manufacture.

4.3 Current BCG Vaccine Formulation

The current BCG vaccine, internationally referred to as the 'freeze-dried BCG vaccine', is a lyophilized powder reconstituted in a diluent prior to intradermal administration [30]. The lyophilized formulation typically contains live *M. bovis* BCG (approximately 2–8 million viable bacteria per mL upon reconstitution) along with sodium L-glutamate monohydrate as a stabilizer [30]. The recommended storage temperature for the formulation is 2–8 °C. The diluent commonly used for reconstitution is sodium chloride and is packaged with the multi-dose vial or ampoule (10 or 20 doses per container). The recommended dose for children under the age of one is 0.05 mL. For those age one year to adult the dose is 0.1 mL. Upon reconstitution, the vial must be kept refrigerated, protected from sunlight, and used within 6 hours or be discarded irrespective of the number of doses used. The concern is that after reconstitution there is a decrease in bacterial viability with time, rendering the vaccine ineffective [31].

4.3.1 BCG Vaccine Strain Variability

The current BCG strains are all descendants of the original *M. bovis* isolate, cultured and passaged by Calmette and Guérin during the period 1909–1921 [32–34]. However, new strains displaying phenotypic and genotypic differences were a result of passaging under different laboratory conditions, prompting the WHO to stock lyophilized seed lots of the vaccine strains [34, 35]. Since 1956, the WHO housed these strains to avoid any further deviations from the original BCG [32]. Amongst the different BCG vaccine strains available, four strains, namely the Danish strain 1331, the French Pasteur stain 1173 P2, the Glaxo strain 1077, and the Tokyo strain 172 account for nearly 90% of the vaccines currently manufactured worldwide [31, 36, 37]. Currently, there is no general consensus about efficacy from one strain to another. Moreover, variable culturing methods and freeze-drying techniques employed by different manufacturers have led to noticeable differences in the number of viable bacteria per vaccine dose [31]. These factors have an impact on the allergenic properties of the vaccine, resulting in implications for both the reactogenicity and for the induction of delayed-type hypersensitivity [38].

4.3.2 BCG Lyophilization for Stability

Live bacterial vaccines are often lyophilized to protect them from environmental stresses such as extreme temperatures and humidity, and to maintain the potency of the formulation [39, 40]. A combination of formulation composition and optimized freeze-drying conditions are required to obtain a stable and viable product. The lyophilization process involves

three main steps: freezing, primary drying (sublimation), and secondary drying (removal of the bound water). These three steps have to be optimized to avoid process stress such as low temperature, crystal formation, and ionic strength changes [39–41]. Moreover, lyophilized formulations further necessitate an optimum control of the residual moisture content in the final product, and appropriate storage conditions such as temperature, humidity, and protection from sunlight; all of which have a significant impact on stability and efficacy [41]. For example, based on the lyophilization and stabilization techniques employed by different manufacturers, differences in the heat stability of BCG vaccine were observed, with the Tokyo strain 172 having a better thermostable profile compared with both the French strain 1172 and the Danish strain 1331 [37, 42]. In addition, the lyophilized formulations incorporate a stabilizer to minimize the detrimental effects such as crystal formation and subzero temperatures, and to preserve potency during the freeze-drying process [39]. Moreover, these formulations require a diluent for reconstitution and parenteral administration. For some of the current BCG vaccines, the stabilizer sodium glutamate makes reconstitution with the diluent more difficult [37, 43]. The diluents vary widely in their composition and are designed specifically for each vaccine [39].

4.3.3 Manufacturing Process

GMP requirements are needed to make safe and effective vaccines [44]. No single facility is able to meet the requirements for the production of all vaccines, therefore facilities must be tailored for each specific vaccine [44]. The manufacturing process for the production of the current BCG vaccine has remained unchanged over the last half-century [45] and there is no standardized production amid manufacturers [46]. One plausible reason for the highly variable protection (0–80%) of the current BCG vaccine may be the failure to have stringent global manufacturing standards [47, 48]. Very few manufacturers of the BCG vaccine are currently able to meet the basic GMP requirements for manufacturing [31]. At present, there are only four manufacturers of the BCG vaccine that are WHO pre-qualified for sale to UN agencies (Table 4.1). The limited number of WHO-qualified manufacturers is concerning, as the loss of even a single manufacturer could have a significant impact on the global BCG vaccine supply [31].

The traditional process of preparing the BCG vaccine is very tedious. The vaccine is produced as surface pellicles in culture flasks requiring months for growth. In addition, bacteria grow as aggregates rather than single bacterial cells, requiring de-agglomeration. The slow growth process limits the opportunity for correction of growth parameters [49, 50]. The *M. bovis* isolate cultures are extremely sensitive to external variations. Factors such as the quality of raw materials, media production, and culture techniques all contribute to the final product variability [51]. For example, the Denmark manufacturer Statens Serum Institut (SSI) experienced a slower growth of BCG during 2008–2010, resulting in varying heat stability and proportion of viable bacteria in the final product. Bacterial growth was stabilized in May 2010 after they changed their glycerol supplier [51].

Accurate quantification and QC is also hampered by an unknown proportion of nonviable bacteria in the vaccine which may have variable effects on the efficacy [43, 49]. After the bacteria are harvested, they are filled into vials or ampoules, excipients are added, and the mixture is freeze-dried [45]. Clements (2003) stated that BCG is produced as a laboratory product resulting in batch-to-batch variability, leading to an inconsistent

Table 4.1 *List of WHO pre-qualified manufacturers of BCG vaccine*

Manufacturer	Country	Packaging	Strain	Formulation	Shelf-life
BB-NCIPD Ltd.	Bulgaria (Distributed by InterVax, Canada)	10/20 Dose ampoules	Russian BCG-I	Lyophilized + 0.9% sodium chloride diluent	36 Months at 2–8 °C, protected from light
Japan BCG Laboratory	Japan	20 Dose ampoules	Tokyo 172-1 strain	Lyophilized + 0.9% sodium chloride diluent	24 Months at 2–8 °C
Serum Institute of India Ltd	India	20 Dose vial	Russian BCG-I	Lyophilized + sodium chloride diluent	24 Months at 2–8 °C
Statens Serum Institut	Denmark	10 Dose vial	Danish strain 1331	Lyophilized + diluent	12–24 Months at 2–8 °C

efficacy profile [31]. A recent example of this complication was the recall of the BCG vaccine by the Australian and New Zealand governments. This was due to the lack of efficient monitoring for environmental contamination at the manufacturing premises resulting in non-sterile vaccines [52, 53]. Moreover, Biering-Sørensen has recently shown that the variations in the BCG vaccine production amongst manufacturers may influence the immunogenicity [51]. The current QC protocol for the BCG vaccine measures the viable bacterial counts and the ability to induce tuberculin sensitivity in the final product [54]. However, these measures are not optimal correlates of protection against *M. tb* [31].

4.3.4 Packing and Storage

Packing and storage processes influence the overall stability of the vaccine and therefore affect efficacy. The current BCG vaccine is filled in amber-colored vials or ampoules, freeze-dried, and sealed under vacuum [31]. Some of the currently supplied vaccines in vials use rubber stoppers and show no loss of stability or viability of the BCG vaccine. There are some contradicting reports about the use of an inert gas for sealing and its effect on vaccine viability [55, 56]. The current BCG vaccines are all recommended for storage at 2–8 °C to maintain their efficacy.

4.3.5 Transportation

The transportation, namely the vaccine supply chain (Figure 4.2), ensures the vaccine travels from the point of production to the individual to be vaccinated while maintaining safety and immunogenicity [57]. Most vaccines, including the current BCG vaccine, are temperature and light sensitive and thus require complex transportation systems. Vaccine instability and loss of potency is a common problem associated with transport and storage [39]. Use of vaccine vial monitors (VVM) is an effective way to determine the loss of potency. VVMs detect exposure to extreme heat conditions and reduce the risk of immunization with impotent vaccines [39]. Cold-chain transport and storage facilities are designed to maintain an environment that promotes the longevity of the vaccines. However, inadequate facilities, lack of communication amongst transport personnel, and multiple storage areas (temporary

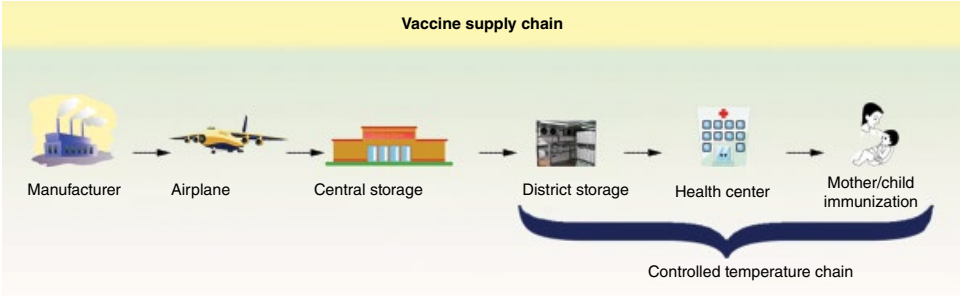


Figure 4.2 *The stages of a vaccine supply chain from the manufacturer to the immunized individual. The introduction of a 'Controlled Temperature Chain' will provide vaccine supply to the remote regions of the world*

airport storage for customs clearance; central, regional, and local storage) increase the risk of degradation [57]. Furthermore, untrained healthcare workers run the risk of misreading the VVMs, therefore administering vaccines that have been exposed to extreme temperatures either during storage or transport [39].

4.3.6 Needle-stick Issues

Given that the current freeze-dried BCG vaccine is to be administered parenterally, the exposure of healthcare workers to needles is inevitable. Needle-stick injuries are a common incident among healthcare workers around the world [58, 59]. However, needle-stick injuries are highest in LMICs where the prevalence of infectious diseases is also the highest [9, 11]. It is estimated that the average African healthcare worker suffers from two to four needle-stick injuries per year due to poor sharps-disposal training [9, 11]. Moreover, children in these areas scavenge used needles to supplement family income and incur accidental needle-stick injuries, and often these injuries go unreported. To minimize the risk of exposure to needle-stick injuries, a safe, needle-free and cost-effective alternative must be developed [10].

4.4 Novel TB Vaccination Strategies

The WHO, together with the European Respiratory Society (ERS), outlined a framework to eliminate TB incidence by 2050 and to prevent its resurgence as a public health problem. Although TB rates are declining, this reduction is not sufficient to reach the globally projected goal of 2050 [60]. The existing BCG formulation has remained largely unchanged for many decades, as mentioned in the previous sections, and continues to provide partial protection against *M. tb*. [61]. In addition, while many novel candidates have shown great promise in clinical trials, none have yet reached the market.

TB vaccines currently being researched are broadly classified under two categories: a) whole-cell vaccines (live attenuated bacterial- and viral-vectored), and b) subunit vaccines [62]. Whole-cell vaccines include modified recombinant BCG, attenuated *M. tb*, and viral-vectored vaccines. Subunit vaccines consist of either a cell wall component, an antigenic protein, DNA, or other immunodominant mycobacterial antigens. Table 4.2 discusses

Table 4.2 *Whole-cell and subunit TB vaccine formulations in preclinical trials*

Vaccine type	Antigens	Adjuvant[a]	Formulation[a]	Route of administration[a]	Animal Model	References
			Whole-cell vaccines			
Live attenuated bacteria	BCG	None	Dry powders	Pulmonary	Guinea pigs	[63]
	BCG	None	Sodium alginate MPs	Oral	Mice	[64]
	BCG	None	Lipid formulation	Oral	Mice	[65]
Viral-vectored	Aeras 402/Crucell Ad35 Ag85A, Ag85B, TB10.4	None	Dry powders	Pulmonary	—	[66]
	MVA (modified vaccinia virus)	None	Nanopatch microneedle	Transdermal	—	[67]
			Subunit vaccines			
Gene- or Antigen- based	Ag85B-MPT-64-Mtb8.4	Incomplete Freund's adjuvant	Chitosan MPs	S.C.	Mice	[68]
	DNA (hsp65)	Trehalose dimycolate	PLGA MPs	I.M.	Guinea pigs, Mice	[69]
	Ag85B	Trehalose dimycolate	PGLA MPs	Pulmonary	Guinea pigs	[70]
	DNA (hsp65)	Trehalose dimycolate	PLGA MPs	I.M.	Mice	[71]
	DNA (Ag85B + MPT64 + MPT83)	Dimethyldioctadecyl-ammonium bromide	PLGA MPs	I.M.	Mice	[72]
	Ag85A	CpG	Poloxamer 407 NPs	Pulmonary	Mice	[73]
	DNA Ag85B	None	Chitosan NPs	S.C.	Mice	[74]
	Ag85A	CpG	Pluronic stabilized NPs	Pulmonary	Mice	[75]
	DNA-HLA-A-0201	None	Chitosan NPs	Pulmonary	—	[76]
	DNA-Rv1733c	None	PLGA-PEI NPs	Pulmonary	Mice	[77]
	Bacteriocins	None	Liposomes	I.V.	Mice	[78]
	Trehalose dibehenate	CAF01	Liposomes	S.C.	Mice	[79]
	DNA (hsp65)	None	Liposomes	Intranasal, I.M.	Mice	[80]
	ESAT-6	CAF01	Liposomes	S.C.	Mice	[81]
	Ag85B-ESAT6	CTA-DD/ISCOMs	Liposomes	Intranasal	Mice	[82]
	RUTI (detoxified cellular fragments of *M. tb*)	None	Liposomes	S.C.	—	[83]

[a] S.C., Subcutaneous; I.M., Intramuscualr; I.V., Intravenous; MPs, Microparticles; NPs, Nanoparticles; ISCOMs, Immunostimulating complexes.

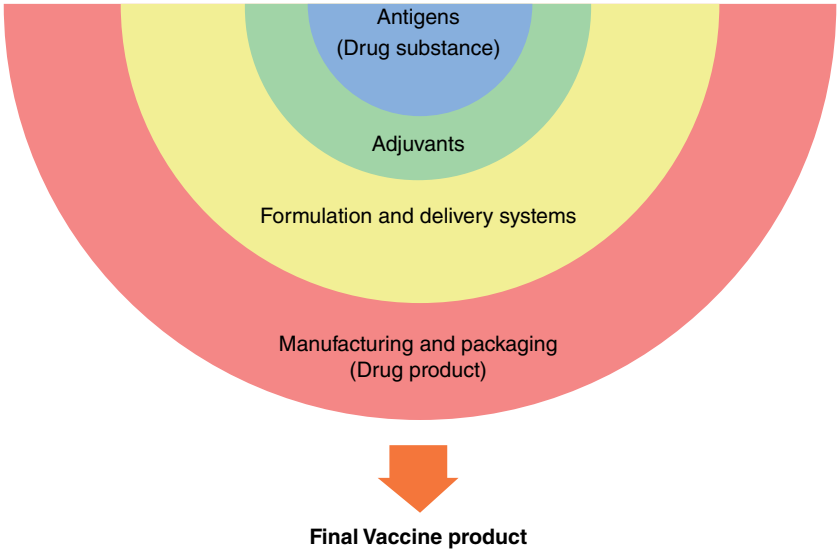


Figure 4.3 *An ideal final vaccine product consists of the right combination of antigens, adjuvants, optimized excipients, and appropriate packaging to ensure the quality and immunogenicity*

whole-cell and subunit TB vaccines that are formulated as delivery systems and that have been evaluated in preclinical animal models. Manufacturing of these vaccines is more complex than for drug compounds and requires sophisticated processes to confirm their safety, antigenicity, and immunogenicity. Therefore, the development of a successful vaccine requires more than just a promising antigen; it involves selection of the right combination of antigens, adjuvants, excipients, and packaging (Figure 4.3).

Here, we discuss formulation and stabilization techniques currently being researched for experimental TB vaccines in preclinical animal models. Novel preparation methods, with a focus on dry powder vaccines and the selection of excipients that provide stability to the antigen, will be briefly discussed. In addition, we deliberate on the large-scale manufacturing processes, role of packaging, and streamlining the regulatory approval processes for new vaccines.

4.4.1 Formulation and Stabilization Techniques

4.4.1.1 Vaccine Formulation

The formulation of a stable vaccine product is based on the nature and complexity of the antigen or whole-cell vaccine. A stable formulation, in its final container or closure system, must maintain physical, chemical, microbiological, therapeutic, and toxicological specifications [84]. Thus, a rational and systematic effort for the physiochemical characterization of the final product needs to be established. The types of analysis required to thoroughly characterize the final formulation will depend on the vaccine type (subunit, viral-vector-, or bacterial-based vaccine). Novel TB vaccines have been formulated as various delivery systems (Table 4.2), including polymeric micro- and nanoparticles, liposomes,

emulsions, and so on, for the purpose of antigen protection and to target specific mucosal cells and tissues. These delivery systems, in conjunction with different routes of delivery, aim to elicit a potent immune response. To further nanoparticulate vaccines, the mechanism of action needs to be evaluated, including a better understanding of their *in vivo* biodistribution and fate. Bivas-Benita *et al*. formulated chitosan nanoparticles containing DNA plasmid encoding *M. tb* epitopes [76]. In addition, they also formulated a poly-(*d,l*-lactide-*co*-glycolide) (PLGA)–polyethyleneimine (PEI) biodegradable polymer-based nanoparticulate vaccine with DNA plasmid encoding *M. tb* latency antigens [77]. In both studies, an increase in interferon-gamma (IFN-γ) secretion was observed with nanoparticulate vaccines compared with the pulmonary delivery of plasmid in solution or the same vaccination given intramuscularly.

Formulating vaccines in a dry powder solid state provides thermostability to the antigen. Accidental freezing of the product significantly affects the vaccine's integrity, especially if it is formulated as a liquid vaccine. Dry powder formulations overcome these thermally critical weak points [85]. Furthermore, a number of drying processes have been developed to formulate dry powders with very low residual moisture (< 3%) to confer thermal stability [86] including spray drying, spray-freeze drying, and supercritical fluid drying [87].

Vaccines in the dry solid state reduce the mobility of macromolecules and eliminate degradation pathways such as hydrolysis. In addition, some bacteria are known to survive under conditions of complete dehydration for years (anhydrobiosis), and can revive and resume growth when water becomes available [88]. However, the drying process for live vaccines, specifically lyophilization, needs to be thoroughly validated as it can cause viability loses and render vaccines ineffective [89]. In addition, lyophilized vaccines currently require a reconstitution step prior to administration. Dry powder solid-state vaccines delivered by the pulmonary route do not require reconstitution, thus avoiding some of the pitfalls discussed later.

4.4.1.2 Adjuvants

Adjuvants are substances added to subunit vaccines to augment the immune response elicited by the antigen. Inclusion of an adjuvant reduces the antigen dose required to modulate the immune response. The antigen, the delivery system, the route of administration and possible side effects determine the choice of adjuvant. Furthermore, the characteristics of an ideal adjuvant are a long shelf-life, safety, stability, cost-effectiveness, and they should not induce an immune response against themselves [87]. The most commonly used adjuvants are alum salts, due to their established safety record.

TB subunit vaccines that induce a Th-1 cellular immune response may require adjuvants such as monophosphoryl lipid A (MPL; a TLR-4 agonist). MPL derived from bacterial lipopolysaccharide is present in many novel adjuvants such as the adjuvant AS01 (liposomal formulation with a saponin), AS02 (water-in-oil emulsion with a saponin), and AS04 (MPL with alum) [90]. In a recent clinical trial, Hybrid 1 subunit vaccine (Ag85 and ESAT-6) was combined with a liposomal adjuvant (CAF01) to induce antigen-specific immune responses three years post immunization [91]. The subunit TB vaccine H4 (TB10.4 and Ag85B fusion protein), when formulated with IC31 adjuvant (induces TLR-9/MyD88 signaling pathway), induced a persistent polyfunctional CD4 T cell response in adults [92]. These studies show the role of adjuvants in subunit TB vaccines.

4.4.1.3 Excipient Selection

The word excipient is derived from the Latin word 'excipere' means 'to except'. In simplified terms an excipient is anything other than the active ingredient [93]. Excipients are selected based on the route of delivery and are added to a formulation to improve its structural integrity, and to extend shelf-life. Over the decades, various excipients have been evaluated to enhance stability. Potential excipients are usually selected on the basis of their established use in other drug and vaccine formulations. Excipient screening for vaccines usually consists of choosing generally regarded as safe (GRAS) materials. However, non-GRAS excipients need to be explored in an attempt to identify one or a combination of excipients to provide stability.

TB vaccines currently being evaluated in preclinical and clinical trials are mostly liquid formulations, and are at risk of chemical and physical degradation. For a liquid product, stability will depend on the solution pH, ionic strength, redox potential, and the temperature at which it is stored. The pH affects the rate of enzymatic activity of live attenuated bacterial and viral vaccines [94]. However, the inclusion of excipients can stabilize vaccines by different mechanisms, including buffering against pH changes, hydration (non-reducing sugars such as trehalose, mannitol, sucrose, etc., and amino acids such as leucine, lysine, glycine, etc.), decreasing adsorption, and aggregation (non-ionic surfactants), and therefore preventing protein interactions due to steric hindrance by different molecular weight polymers and serum albumins.

A number of physicochemical assays including absorbance and fluorescence spectroscopy, circular dichroism, glass transition temperature (Tg), etc. are used to monitor changes to antigen and excipients undergoing stresses of temperature, salt, and pH [95]. The Tg is defined as the temperature below which the molecular mobility of the product (consisting of any bound water, antigen, and excipients) approaches zero. Tg is usually determined using differential scanning calorimetry. Tg is important to preserve the amorphous nature of a dry powder solid-state vaccine. A high Tg is thus critical to ensure that the final product remains amorphous and maintains efficacy. Excipients that promote high Tg are ideal. Jin *et al*. formulated adenoviral-based TB vaccines using a combination of excipients (mannitol, trehalose, cyclodextrin, and dextran) to produce a Tg of >97 °C after spray drying [66].

4.4.1.4 Alternative Route of Delivery

The intradermal route is the current route of BCG vaccine administration. However, this may not be the optimum route to elicit a strong immune response against TB, as the disease is mostly transmitted as droplet aerosols. An ideal route of delivery mimics the entry portal of the pathogen. Alternative delivery routes such as the pulmonary and sublingual routes target immune cells that are critical to generate an adaptive and long-term memory immune response in the host. Needle-free routes of delivery are more patient compliant and can be self-administered. Moreover, needle-free routes of administration eliminate the risks associated with parenteral vaccination [96].

4.4.1.5 Thermostability

A thermostable vaccine does not require cold-chain storage and maintains potency for extended periods when exposed to extreme temperatures. The importance of thermostable vaccines was first demonstrated during the global eradication of smallpox. Leslie Collier was credited with developing a "heat-stable" smallpox vaccine using novel excipients in conjunction with the lyophilization process, allowing the vaccine to be stable at 37 °C [97].

The WHO initiated the global smallpox eradication campaign in 1967, leading to complete eradication of smallpox in 1980 [98].

Most vaccines currently in the market require continuous cold-chain storage (2–8 °C) to remain effective. The temperature in some tropical countries can exceed 45 °C on a hot summer day. Therefore, thermal stability is important for the manufacture, distribution, and administration of vaccines. A loss in potency can also occur due to freezing conditions. Failure to maintain regulated temperature conditions can be observed in the supply chain from the point of production (usually in high- and middle-income countries) to the point of administration (usually in LMICs). At present, one in five children do not receive some vaccines under the Expanded Program on Immunization (EPI), due to the absence of a robust cold-chain system [99].

Future vaccines need to be stable at extreme temperatures to ensure better product stability leading to a longer shelf-life. However, achieving stability at high temperatures even for a short period of time could be significant. This has been shown by the success of the MenAfriVac™ vaccine which can withstand temperatures up to 40 °C for 4 days [100]. The "Controlled Temperature Chain" (CTC) becomes important during the final levels of the supply chain when cold-chain failure is most likely to occur (Figure 4.2). An ideal vaccine has a two year shelf-life under cold-chain management and another few days at CTC, allowing immunization outreach programs to reach remote regions [101]. Wong *et al*. spray dried BCG with leucine as an excipient and demonstrated four months stability at 25 °C [102]. Price and colleagues recently improved on the spray dried BCG formulation by Wong *et al*., using a factorial design of excipients to generate a BCG dry powder which shows stability at high, ambient, and freezing temperatures for over 6 months [103]. Jin *et al*. spray dried adenovirus-vectored vaccine (Aeras 402) using non-reducing sugars, and viral stability was observed for 12 months at 4 °C and 25 °C, and five weeks at 37 °C [66]. Kunda *et al*. has recently spray dried a live bacterial vaccine with non-reducing sugars that showed stability when stored at 25 °C (12 months) and 40 °C (6 months) [104]. In addition, work from the same laboratory reported a spray dried virus-like particle-based vaccine which achieved one year of stability at 37 °C [105]. These studies show that a dry powder solid-state formulation provides enhanced thermostability compared with the traditional liquid formulation.

4.4.2 Manufacturing of TB Vaccines

Downstream product development is a critical manufacturing aspect often neglected during the initial design and development process (Figure 4.4). Development and manufacturing must be well integrated to achieve a successful final product. Future strategies should include altering manufacturing to suit each antigen type to ensure consistency [106]. The manufacturing process is a complex and capital-intensive endeavor.

Inconsistencies in the production processes, storage, and transportation result in a final product having variable immunogenicity. Future manufacturing must follow globally defined and validated protocols, using strict QC processes to ensure consistent production from batch to batch. GMP and Chemistry Manufacturing and Controls (CMC) information are critical for filing of an investigational new drug (IND) to be evaluated in clinical trials. The CMC includes complete information on characterization, manufacturing processes, and analytical assays used to identify purity, quality, safety, and stability data [107].

The requirements associated with setting up new manufacturing facilities specific for each vaccine are cost-intensive. Any increase in cost will impact vaccine procurement and

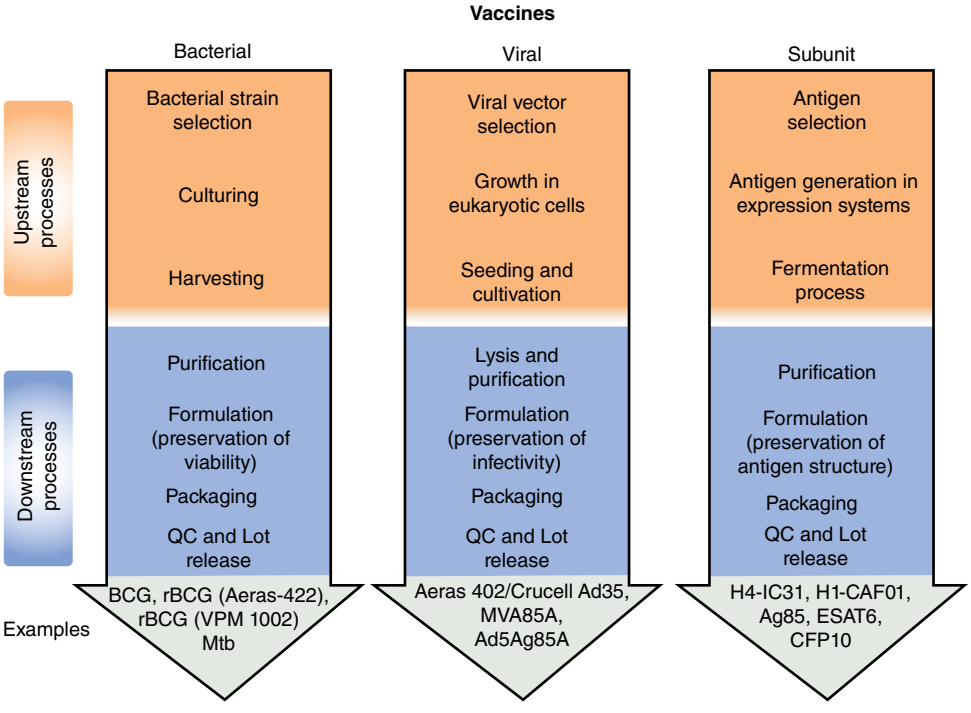


Figure 4.4 *The upstream and downstream processes involved in the preparation of a successful final vaccine product. The upstream research and development should be integrated with the downstream manufacturing and packaging activities*

distribution in LMICs. In addition, investment in new manufacturing facilities is a risk for pharmaceutical industries because of the high failure rates associated with investigational vaccine candidates. However, modular or small-scale manufacturing facilities can be rapidly constructed, thereby reducing the capital required to mitigate failure risk. Modularization of manufacturing facilities has the potential to shorten the development process and enable faster throughput of candidates for proof-of-concept clinical trials [44]. Manufacturing can play an important role in the overall success of the final product in regions of the world where it is needed the most.

4.4.3 Whole-Cell Vaccine

4.4.3.1 Live Bacterial Vaccines

Live bacterial vaccines have the simplest manufacturing processes of the three types mentioned here. Production of bacterial vaccines includes cultivation of bacteria, harvesting, and sometimes drying the bacterial suspension into a final product for long-term stability. The history of manufacturing BCG in the last century, and other recombinant live mycobacterial strains evaluated recently in clinical trials, should help with the standardization of future manufacturing [108]. Mycobacterial culture requires long growth periods, leading to limited opportunity to correct any growth parameters. Novel methods to enumerate bacterial viability and potency in shorter time frames need to be developed. Such methods will

be critical for recombinant live mycobacterial vaccines. Current assays of potency used in BCG manufacturing could be modified and incorporated into potency tests of other novel live mycobacterial vaccines. The ratio of live to dead bacteria in a final product is an important determinant of potency, and the dead bacterial population may cause undesirable side effects; thus a reliable viability marker would be useful for the assessment of the final product. Other measurements of potency, such as the level of expression of recombinant proteins, must be validated and documented.

4.4.3.2 Live Viral Vaccines

Viral vectors are designed to express subunit antigens. Recombinant viral vectors can clone multiple immunodominant antigens and can be manufactured in high titers, thus allowing for easy scale-up. Few virally expressed subunit vaccines are currently in clinical trials. Most recently, Modified Vaccinia virus Ankara (MVA), a replication-deficient strain of Vaccinia virus, was used as a viral vector for Ag85A in Phase 2b trials in South Africa [109]. The MVA vector is designed to undergo one infection cycle in human cells to ensure safety [110].

The manufacturing of viral vectors involves propagation in suitable cell lines. Viral vaccines have historically been mass produced in embryonated chicken eggs and conventional cell substrates such as chicken embryo fibroblasts. However, these manufacturing methods quickly reach capacity limits for production. More recent production techniques include cell substrates of avian or human origin to potentially overcome the production limits of the former substrates as they are highly replication-proficient. Other advantages of these production techniques include ease of scale-up, rapid response to pandemic situations, and overcoming problems related to egg-related allergies. Optimization of cell substrate growth conditions, such as choice of growth medium and long-term cultivation conditions, and so forth, are critical for consistent safety and potency. Other growth parameters, such as stability during culture, purity, contamination with adventitious and endogenous agents, and oncogenicity will be important in the manufacture of viral vaccines.

4.4.4 Subunit Vaccines

Subunit vaccines against TB consist of either antigenic proteins, DNA, lipids, or carbohydrates. Subunit vaccines are advantageous over live vaccines due to their safety, ease of manufacture, and the ability to generate specific immune responses in the vaccinated host. Adjuvants can potentiate immune responses through interaction with toll-like receptors (TLRs) [111]. In order to elicit an appropriate immune response, antigens must resist conformational changes during the manufacturing processes. Furthermore, protein-based subunit vaccines must have high levels of purity to prevent side-effects usually associated with whole-cell vaccines. Subunit purification can also be accomplished using platform technology, chromatographic adsorbents, suspended absorption, and filtration [112].

4.4.5 Regulatory Approval Process

LMICs rely on the FDA and the European Medicines Agency (EMEA) for the regulatory approval of vaccines. However, there are no clear global regulatory pathways in place for novel TB vaccines. Global regulatory mechanisms can be streamlined with some effort, since there are many common approaches between countries to manufacture, test, and license. For this to happen, a continuous dialogue between the developers and regulatory

agencies across countries, at the early stages of development, is critical. Furthermore, flexible regulatory steps to reduce vaccine-approval timelines are needed.

Bacterial vaccines are being tested in preclinical and clinical trials. Lessons learned from current BCG vaccine manufacturers will aid in the regulatory approval of new candidates. However, these products will require thorough characterization, including viability and immunogenicity testing. The degree of bacterial attenuation, and the persistence of the bacteria in the host, must be documented for whole-cell vaccines, whether based on attenuated *M. tb* or BCG strains. In addition, any product development with whole-cell vaccines, including stabilizing by drying, needs to be tested for changes in viability and immunogenicity.

Since the 2000s, viral-vectored vaccines have undergone stringent regulatory approval processes. A recent detection and subsequent elimination of potentially adventitious viruses from a viral-vaccine-production facility prompted this change in the regulatory approval process [113]. Individuals were accidently exposed to Simian Virus 40 (SV40) via a contaminated polio vaccine manufactured in monkey kidney cells [114]. The SV40 contaminant went undetected during the manufacturing process.

For a subunit vaccine, the adjuvant and the antigen combination needs to be approved by the regulatory agency, since an adjuvant is not the active ingredient. A justification for the use of an adjuvant is also required, taking into account the preclinical and early clinical studies comparing adjuvanted and non-adjuvanted vaccines. Adjuvanted vaccines may require a complex and lengthy regulatory approval process compared with non-adjuvanted vaccines. Assessment of serious adverse events (SAEs), potential autoimmune-related adverse events, and inflammatory-mediator-related events should be required for approval [115]. The approval processes of TB vaccines need to be harmonized among the FDA, the EMEA, and the International Conference on Harmonization (ICH), since no current consensus exists.

4.4.6 Vaccine Packaging

Appropriate packaging of the final product ensures long-term stability during storage and transportation. Precise wording on the package inserts and labeling according to the GMP regulations certifies correct use of the final product. A dry powder formulation to be administered parenterally requires reconstitution using diluent. Although reconstitution appears to be a straightforward process, errors can occur when healthcare workers are not adequately trained. Incorrect volumes of diluent may be used, sterility may be compromised, and multi-dose vials may be contaminated as doses are withdrawn. Furthermore, vaccines that require reconstitution involve greater packaging volume to facilitate the inclusion of the diluent, leading to increased costs. In addition, it causes logistical considerations due to limited cold-chain storage space. Vaccine manufacturers should consider early integration of development processes in conjunction with the product profile recommendations of the Vaccine Presentation and Packaging Advisory Group (VPPAG) and the WHO guidelines on programmatic suitability of vaccine candidate approval [116, 117].

4.5 Future Perspective

Ideal future TB vaccines should have enhanced stability during storage and transportation, compact packaging, and be easily administered to the population without the need for highly trained medical personnel. These characteristics are crucial for the overall success of

new vaccines. More importance should be given to the physical and chemical interactions between the antigen and the excipients. Excursions from the recommended storage conditions exacerbate interactions affecting the potency and safety. The implementation of CTC, mainly at the final steps of the supply chain, could significantly reduce vaccine waste and facilitate distribution to remote regions. Non-GRAS excipients that may provide potential stability to novel TB vaccines should be explored. Dry powder formulations utilizing novel preparation techniques, such as spray drying in combination with the pulmonary delivery route, should be explored with more vigor. To avoid contamination, manufacturing facilities should be tailored specifically for each vaccine. Modularization of manufacturing will make vaccines affordable to the purchaser. This could have a huge impact on LMICs where TB is most prevalent.

4.6 Conclusions

Research efforts in vaccine development against TB continue, with many promising new candidates and immunization regimens in clinical trials. However, the formulation side of development remains largely unchanged since lyophilization of BCG in the early 1960s. Over half of the world has BCG immunization programs in place, but many of these programs remain unsuccessful. This is illustrated by startling infection rates and dismal mortality statistics. While most of the failures of BCG can be explained by problems with the bacteria themselves, a significant portion may be due to BCG vaccines that are rendered ineffective by improper storage conditions and poor administration techniques. In an era where so many vaccines against other infectious diseases are mosaics of antigens, adjuvants, stabilizers, immune-modulators, and in some cases nanocarriers, this is unacceptable.

Formulation and manufacture must be re-envisioned alongside antigen discovery, whether the vaccine uses live bacteria, antigenic proteins, or viral-vectors as a platform for immune-stimulation. It is important to remember that the majority of TB-burdened countries are somewhat rural, swelteringly hot, and resource-poor. This translates to unfavorable storage conditions and poorly trained volunteers administering vaccines, which can have large effects on the efficacy of the vaccine. These conditions must be considered during the design and preparation process to ensure that every person immunized receives a potent, safe, and effective vaccine.

Uniformity in the antigen and formulation, the route of administration, the manufacturing conditions, the storage, and transportation profiles should be a worldwide goal. This can and should be implemented even at present with current BCG immunization programs. Lessons learned from the current vaccine preparation will inform future TB vaccine preparation. These lessons are critical for successful mass immunization.

References

[1] Centers for Disease Control and Prevention (2011) A CDC framework for preventing infectious diseases: sustaining the essentials and innovating for the future, Atlanta, GA.
[2] Centers for Disease Control and Prevention (2013) Understanding How Vaccines Work, Atlanta, GA.
[3] Newsted, D., Fallahi, F., Golshani, A. and Azizi, A. (2015) Advances and challenges in mucosal adjuvant technology, *Vaccine*, **33**, 2399–2405.

[4] Lee, S. and Nguyen, M.T. (2015) Recent advances of vaccine adjuvants for infectious diseases, *Immune Netw.*, **15**, 51–57.
[5] Gellin, B.G. and Salisbury, D.M. (2015) Communicating the role and value of vaccine adjuvants, *Vaccine*, **33** Suppl. 2, B44–B46.
[6] Gupta, A., Das, S., Schanen, B. and Seal, S. (2015) Adjuvants in micro- to nanoscale: current state and future direction, *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.*, **8**(1): 61–84.
[7] Vehring, R. (2008) Pharmaceutical Particle Engineering via Spray Drying, *Pharm. Res.*, **25**, 999–1022.
[8] Vaccines and Biologicals World Helath Organization, Department of Immunization (2005) Monitoring vaccine wastage at country level: Guidelines for programme managers.
[9] Wilburn, S.Q. (2004) Needlestick and Sharps Injury Prevention, *Online J. Issues Nurs.*, **9**, 5.
[10] Pruss-Ustun, A., Rapiti, E. and Hutin, Y. (2005) Estimation of the global burden of disease attributable to contaminated sharps injuries among health-care workers., *Am. J. Ind. Med.*, **48**, 482–490.
[11] Wilburn, S.Q. and Eijkemans, G. (2004) Preventing needlestick injuries among healthcare workers: a WHO-ICN collaboration., *Int. J. Occup. Environ. Health*, **10**, 451–456.
[12] Calmette, A., Guerin, G. and M. Breton, M. (1907) Contribution a l'etude de la tuberculose experimental du cobaye (infection et essais de vaccination par la voie digestive), *Ann. Inst. Pasteur*, **21**, 401–416.
[13] Calmette, A., Boquet, A. and Negre, L. (1921) Contribution a l'etude du bacille tuberculeux bilie, *Ann. Inst. Pasteur*, **35**, 561–570.
[14] Calmette, A. and Guerin, C. (1920) Nouvelles recherches experimentales sur la vaccination des bovides contre la tuberculose, *Ann. Inst. Pasteur*, **34**, 553–560.
[15] Van Deinse, F. and Sénéchal, F. (1950) BCG on Sauton's medium: Effect of a long series of subcultures on the morphological and biological properties of BCG cultures, *Bull. World Health Organ.*, **2**, 347–354.
[16] Plotkin, S. (2011) History of vaccine development, Springer Science & Business Media.
[17] Gheorghiu, M., Augier, J. and Lagrange, P.H. (1983) Maintenance and control of the French BCG strain 1173-P2 (primary and secondary seed-lots), *Bull. Inst. Pasteur*, **81**, 281–288.
[18] McShane, H. (2011) Tuberculosis vaccines: beyond bacille Calmette–Guérin, *Philos. Trans. R. Soc. B Biol. Sci.*, **366**, 2782–2789.
[19] Benévolo-de-Andrade, T.C., Monteiro-Maia, R., Cosgrove, C. and Castello-Branco, L.R.R. (2005) BCG Moreau Rio de Janeiro - An oral vaccine against tuberculosis – Review, *Mem. Inst. Oswaldo Cruz (Rio de Janeiro)*, **100**, 459–465.
[20] Corbel, M.J., Fruth, U., Griffiths, E. and Knezevic, I. (2004) Report on a WHO consultation on the characterisation of BCG strains, Imperial College, London, 15–16 December 2003, *Vaccine*, **22**, 2675–2680.
[21] Bonah, C. (2005) The "experimental stable" of the BCG vaccine: safety, efficacy, proof, and standards, 1921–1933, *Stud. Hist. Philos. Sci. Part C Stud. Hist. Philos. Biol. Biomed. Sci.*, **36**, 696–721.
[22] Weiss, D.W. (1959) Vaccination Against Tuberculosis with Nonliving Vaccines 1: I. The Problem and Its Historical Background, *Am. Rev. Respir. Dis.*, **80**, 340–358.
[23] Hoft, D.F. and Gheorghiu, M. (1996) Mucosal immunity induced by oral administration of Bacille Calmette-Guerin, Mucosal Vaccines, Academic Press, San Diego, California, pp. 269–279.
[24] Sakula, A. (1983) BCG: who were Calmette and Guerin?, *Thorax*, **38**, 806–812.
[25] Gheorgiu, M. (2011) Antituberculosis BCG vaccine: lessons from the past, in S.A. Plotkin (Ed.), History of Vaccine Development, Springer, New York, pp. 47–55.
[26] Monteiro-Maia, R. and de Pinho, R.T. (2014) Oral bacillus Calmette-Guérin vaccine against tuberculosis: why not?, *Mem. Inst. Oswaldo Cruz.*, **109**, 838–845.
[27] Mortatti, R.C., Maia, L.C.S. and Fonseca, L.S. (1987) Absorption of Mycobacterium bovis BCG administered by the oral route, *Vaccine*, **5**, 109–114.
[28] Clark, S.O., Kelly, D.L.F., Badell, E. et al. (2010) Oral delivery of BCG Moreau Rio de Janeiro gives equivalent protection against tuberculosis but with reduced pathology compared to parenteral BCG Danish vaccination, *Vaccine*, **28**, 7109–7116.

[29] Brasil. Ministério da Saúde (1994) Coordenação Nacional de Pneumologia Sanitária. Segundo informe técnico sobre vacinação/revacinação BCG. Brasília: Centro de Documentação.
[30] World Health Organization (2013), Annex 3: Recommendations to assure the quality, safety and efficacy of BCG vaccines.
[31] Clements, C.J. (2003) Vaccination - The Current Status of BCG, in: S. Kaufmann and H. Hahn (eds.), Mycobacteria and TB, Issues Infect. Dis., Basel, Karger, pp. 46–66.
[32] World Health Organization (2004), Weekly epidemiological record, Geneva.
[33] Locht, C. (2010) The history of BCG, in: N.M. Nor, A. Acosta and S. Elena (eds), The Art and Science of Tuberculosis Vaccine Development, 2nd edn, Oxford University Press, pp. 1–24.
[34] Luca, S. and Mihaescu, T. (2013) History of BCG Vaccine, *Mædica*, **8**, 53–58.
[35] Behr, M.A. (2002) BCG — different strains, different vaccines?, *Lancet Infect. Dis.*, **2**, 86–92.
[36] Abou-Zeid, C., Rook, G.A.W., Minnikin, D.E. *et al*. (1987) Effect of the method of preparation of Bacille Calmette-Guérin (BCG) vaccine on the properties of four daughter strains, *J. Appl. Bacteriol.*, **63**, 449–453.
[37] Quality Safety and Standards (QSS) Team at the World Health Organization (2006), Temperature Sensitivity of Vaccines.
[38] Edwards, L., Palmer, C. and Magnus, K. (1955) BCG Vaccination: Studies by the WHO Tuberculosis Research Office, Copenhagen, *Bull. Med. Libr. Assoc.*, **43**, 174–175.
[39] Kumru, O.S., Joshi, S.B., Smith, D.E. *et al*. (2014) Vaccine instability in the cold chain: mechanisms, analysis and formulation strategies, *Biologicals*, **42**, 237–259.
[40] Rexroad, J., Wiethoff, C.M., Jones, L.S. and Middaugh, C.R. (2002) Lyophilization and the Thermostability of Vaccines, *Cell Preserv. Technol.*, **1**, 91–104.
[41] Adams, G.D. (2003) Lyophilization of Vaccines, in: A. Robinson, M. Hudson and M. Cranage (eds.), Vaccine Protocols. SE - 14, Humana Press, pp. 223–243.
[42] Janaszek, W. (1994) The comparative assays of BCG vaccines of Polish, Danish and Japanese production - laboratory tests, *Przeglad Epidemiol*., **48**, 285–292.
[43] Milstien, J.B. and Gibson, J.J. (1990) Quality control of BCG vaccine by WHO: a review of factors that may influence vaccine effectiveness and safety, *Bull. World Health Organ.*, **68**, 93–108.
[44] Pralong, A., Levine, H.L., Lilja, J. *et al*. (2014) Paradigm shift for vaccine manufacturing facilities: The next generation of flexible, modular facilities, *Eng. Life Sci.*, **14**, 244–253.
[45] Collioud, A., Rothen, S.A. and Dietrich, G. (2008) Developing and Manufacturing Attenuated Live Bacterial Vaccines, BioPharm *Int. Suppl*., 6–14.
[46] V. and B. Global Vaccine Safety (2012), Immunization, Information sheet observed rate of vaccine reactions Bacille Calmette-Guérin (BCG) vaccine, Geneva.
[47] Fine, P.E. and Rodrigues, L.C. (1990) Modern vaccines. Mycobacterial diseases, *Lancet*, **335**, 1016–1020.
[48] Andersen, P. and Doherty, T.M. (2005) The success and failure of BCG - implications for a novel tuberculosis vaccine, *Nature Rev. Microbiol.*, **3**, 656–662.
[49] Levy, F.M., Mande, R., Conge, G. *et a*l. (1968) Perspectives for BCG standardization, *Bibl. Tuberc.*, **24**, 63–190.
[50] Dietrich, G., Mollenkopf, H.-J., Weber, H. *et al*. (2002) Cultivation of Mycobacterium bovis BCG in bioreactors, *J. Biotechnol.*, **96**, 259–270.
[51] Biering-Sørensen, S., Jensen, K.J., Aamand, S.H. *et al*. (2015) Variation of growth in the production of the BCG vaccine and the association with the immune response. An observational study within a randomised trial, *Vaccine*, **33**, 2056–2065.
[52] Department of Health (2012), Tuberculosis (BCG) vaccine - Urgent medicine recall, Australian Government.
[53] Ministry of Health (2012), Tuberculosis BCG Vaccine Recall, New Zealand Government.
[54] World Health Organization (1977), WHO-sponsored international quality control of BCG vaccine. WHO Technical Report Series 8.
[55] Freudenstein, F. (1978) Successful stabilization of BCG vaccines in ampoules sealed under protective gas, *J. Biol. Stand.*, **6**, 243–253.
[56] Bhushan, K., Baily, G.V., Nair, S.S. *et al*. (1975) Freeze dried BCG vaccine sealed in presence of nitrogen, *Indian J. Med. Res.*, **63**, 1335–1343.

[57] Kaufmann, J.R., Miller, R. and Cheyne, J. (2011) Vaccine Supply Chains Need To Be Better Funded And Strengthened, Or Lives Will Be At Risk, *Health Aff.*, **30**, 1113–1121.
[58] Yacoub, R., Al Ali, R., Moukeh, G. et al. (2010) Hepatitis B Vaccination Status and Needlestick Injuries Among Healthcare Workers in Syria, *J. Glob. Infect. Dis.*, **2**, 28–34.
[59] Penalver, J. (2005) Clinical Pearl: Management of Needlestick Injuries in the Health Care Setting, *Ethics J. Am. Med. Assoc.*, **7**, 4.
[60] Smith, R. (2009) Eradication of tuberculosis by 2050 impossible without new vaccine, *BMJ*, 338.
[61] Kamala, T., Paramasivan, C.N., Herbert, D. *et al*. (1994) Isolation and identification of environmental mycobacteria in the Mycobacterium bovis BCG trial area of South India, *Appl. Environ. Microbiol.*, **60**, 2180–2183.
[62] Rowland, R. and McShane, H. (2014) Tuberculosis vaccines in clinical trials, *Expert Rev. Vaccines*, **10**, 645–658.
[63] Garcia-Contreras, L., Wong, Y.-L., Muttil, P. *et al*. (2008) Immunization by a bacterial aerosol, *Proc. Natl. Acad. Sci. USA*, **105**, 4656–4660.
[64] Ajdary, S., Dobakhti, F., Taghikhani, M. *et al*. (2007) Oral administration of BCG encapsulated in alginate microspheres induces strong Th1 response in BALB/c mice, *Vaccine*, **25**, 4595–4601.
[65] Aldwell, F.E., Keen, D.L., Parlane, N.A. *et al*. (2003) Oral vaccination with Mycobacterium bovis BCG in a lipid formulation induces resistance to pulmonary tuberculosis in brushtail possums, *Vaccine*, **22**, 70–76.
[66] Jin, T.H., Tsao, E., Goudsmit, J. *et al*. (2010) Stabilizing formulations for inhalable powders of an adenovirus 35-vectored tuberculosis (TB) vaccine (AERAS-402), *Vaccine*, **28**, 4369–4375.
[67] Pearson, F.E. and Kendall, M.A. (2013) Dry-Coated Live Viral Vector Vaccines Delivered by Nanopatch Microprojections Retain Long-Term Thermostability and Induce Transgene-Specific T Cell Responses in Mice, *PLoS One*, **8**, e67888.
[68] dong Zhu, B., qing Qie, Y., ling Wang, J. *et al*. (2007) Chitosan microspheres enhance the immunogenicity of an Ag85B-based fusion protein containing multiple T-cell epitopes of Mycobacterium tuberculosis, *Eur. J. Pharm. Biopharm.*, **66**, 318–326.
[69] de Paula, L., Silva, C.L., Carlos, D. *et al*. (2007) Comparison of different delivery systems of DNA vaccination for the induction of protection against tuberculosis in mice and guinea pigs, *Genet. Vaccines Ther.*, **5**, 2–9.
[70] Lu, D., Garcia-Contreras, L., Muttil, P. *et al*. (2010) Pulmonary Immunization Using Antigen 85-B Polymeric Microparticles to Boost Tuberculosis Immunity, *AAPS J.*, **12**, 338–347.
[71] Coelho, E.A., Tavares, C.A., Lima, K.M. *et al*. (2006) Mycobacterium hsp65 DNA entrapped into TDM-loaded PLGA microspheres induces protection in mice against Leishmania (Leishmania) major infection, *Parasitol. Res.*, **98**, 568–575.
[72] Cai, H., Hu, X.D., Yu, D.H. *et al*. (2005) Combined DNA vaccine encapsulated in microspheres enhanced protection efficacy against Mycobacterium tuberculosis infection of mice, *Vaccine*, **23**, 4167–4174.
[73] Todoroff, J., Ucakar, B., Inglese, M. *et al*. (2013) Targeting the deep lungs, Poloxamer 407 and a CpG oligonucleotide optimize immune responses to Mycobacterium tuberculosis antigen 85A following pulmonary delivery, *Eur. J. Pharm. Biopharm.*, **8**, 40–48.
[74] Meerak, J., Wanichwecharungruang, S.P. and Palaga, T. (2013) Enhancement of immune response to a DNA vaccine against Mycobacterium tuberculosis Ag85B by incorporation of an autophagy inducing system, *Vaccine*, **31**, 784–790.
[75] Ballester, M., Nembrini, C., Dhar, N. *et al*. (2011) Nanoparticle conjugation and pulmonary delivery enhance the protective efficacy of Ag85B and CpG against tuberculosis, *Vaccine*, **29**, 6959–6966.
[76] Bivas-Benita, M., van Meijgaarden, K.E., Franken, K.L.M.C. *et al*. (2004) Pulmonary delivery of chitosan-DNA nanoparticles enhances the immunogenicity of a DNA vaccine encoding HLA-A*0201-restricted T-cell epitopes of Mycobacterium tuberculosis, *Vaccine*, **22**, 1609–1615.
[77] Bivas-Benita, M., Lin, M.Y., Bal, S.M. *et al*. (2009) Pulmonary delivery of DNA encoding Mycobacterium tuberculosis latency antigen Rv1733c associated to PLGA–PEI nanoparticles enhances T cell responses in a DNA prime/protein boost vaccination regimen in mice, *Vaccine*, **27**, 4010–4017.

[78] Sosunov, V., Mischenko, V., Eruslanov, B. *et al*. (2007) Antimycobacterial activity of bacteriocins and their complexes with liposomes, *J. Antimicrob. Chemother.*, **59**, 919–925.
[79] Agger, E.M., Rosenkrands, I., Hansen, J. *et al*. (2008) Cationic liposomes formulated with synthetic mycobacterial cordfactor (CAF01): a versatile adjuvant for vaccines with different immunological requirements, *PLoS One*, **3**, e3116.
[80] Rosada, R.S., de la Torre, L.G., Frantz, F.G. *et al*. (2008) Protection against tuberculosis by a single intranasal administration of DNA-hsp65 vaccine complexed with cationic liposomes, *BMC Immunol.*, **9**, 38.
[81] Aagaard, C., Hoang, T.T., Izzo, A. *et al*. (2009) Protection and polyfunctional T cells induced by Ag85B-TB10. 4/IC31 against Mycobacterium tuberculosis is highly dependent on the antigen dose, *PLoS One*, **4**, e5930.
[82] Andersen, C.S., Dietrich, J., Agger, E.M. *et al*. (2007) The combined CTA1-DD/ISCOMs vector is an effective intranasal adjuvant for boosting prior Mycobacterium bovis BCG immunity to Mycobacterium tuberculosis, *Infect. Immun.*, **75**, 408–416.
[83] Gil, O., Vilaplana, C., Guirado, E. *et al*. (2008) Enhanced gamma interferon responses of mouse spleen cells following immunotherapy for tuberculosis relapse, *Clin. Vaccine Immunol.*, **15**, 1742–1744.
[84] Brandau, D.T., Jones, L.S., Wiethoff, C.M. *et al*. (2003) Thermal stability of vaccines, *J. Pharm. Sci.*, **92**, 218–231.
[85] Wirkas, T., Toikilik, S., Miller, N. *et al*. (2007) A vaccine cold chain freezing study in PNG highlights technology needs for hot climate countries, *Vaccine*, **25**, 691–697.
[86] Burke, C.J., Hsu, T.-A. and Volkin, D.B (1999) Formulation, stability, and delivery of live attenuated vaccines for human use, *Crit. Rev. Ther. Drug Carr. Syst.*, **16**(1), 1–83.
[87] Kunda, N.K., Somavarapu, S., Gordon, S.B. *et al*. (2013) Nanocarriers Targeting Dendritic Cells for Pulmonary Vaccine Delivery, *Pharm. Res.*, **30**, 325–341.
[88] Crowe, J.H., Crowe, L.M., Oliver, A.E. et al. (2001) The trehalose myth revisited: introduction to a symposium on stabilization of cells in the dry state, *Cryobiology*, **43**, 89–105.
[89] Corbel, M.J. (1995) Reasons for instability of bacterial vaccines, *Dev. Biol. Stand.*, **87**, 113–124.
[90] Mbow, M.L., De Gregorio, E., Valiante, N.M and Rappuoli, R. (2010) New adjuvants for human vaccines, *Curr. Opin. Immunol.*, **22**, 411–416.
[91] Henson, D., van Dissel, J., Joosten, S. *et al*. (2014) Vaccination with a Hybrid 1 (H1) fusion protein combined with a liposomal adjuvant (CAF01) induced antigen specific T-cells 3 years post vaccination in a human clinical trial. (VAC7P. 971), *J. Immunol.*, **192 (Suppl.** 1), 141.16.
[92] Geldenhuys, H., Mearns, H., Miles, D.J.C. *et al*. (2015) The tuberculosis vaccine H4: IC31 is safe and induces a persistent polyfunctional CD4 T cell response in South African adults: A randomized controlled trial, *Vaccine*, **33**, 3592–3599.
[93] Haywood, A. and Glass, B. (2011) Pharmaceutical excipients – where do we begin?, *Aust. Prescr.*, **34**, 112–114.
[94] Newman, J.F.E., Tirrell, S., Ullman, C. *et al*. (1995) Stabilising oral poliovaccine at high ambient temperatures, *Vaccine*, **13**, 1431–1435.
[95] Peek, L.J., Brandau, D.T., Jones, L.S *et al*. (2006) A systematic approach to stabilizing EBA-175 RII-NG for use as a malaria vaccine, *Vaccine*, **24**, 5839–5851.
[96] Mitragotri, S. (2005) Immunization without needles, *Nature Rev. Immunol.*, **5**, 905–916.
[97] Collier, L.H. (1955) The development of a stable smallpox vaccine, *J. Hyg. (London)*, **53**, 76–101.
[98] Breman, J.G. and Arita, I. (1980) The confirmation and maintenance of smallpox eradication, *N. Engl. J. Med.*, **303**, 1263–1273.
[99] World Health Organization (2015), Global vaccination targets "off-track" warns WHO.
[100] Lydon, P., Zipursky, S., Tevi-Benissan, C. *et al*. (2014) Economic benefits of keeping vaccines at ambient temperature during mass vaccination: the case of meningitis A vaccine in Chad, *Bull. World Health Organ.*, **92**, 86–92.
[101] Chen, D. and Zehrung, D. (2013) Desirable attributes of vaccines for deployment in low-resource settings, *J. Pharm. Sci.*, **102**, 29–33.
[102] Wong, Y.-L., Sampson, S., Germishuizen, W.A. *et al*. (2007) Drying a tuberculosis vaccine without freezing, *Proc. Natl. Acad. Sci. USA*, **104**, 2591–2595.

[103] Price, D.N., Kunda, N.K., Ellis, R. and Muttil, P. Design and optimization of a temperature-stable dry powder BCG vaccine. Manuscr Prep.
[104] Kunda, N.K., Wafula, D., Tram, M., Wu, T.H. and Muttil. P. (2016) A stable live bacterial vaccine, *Eur J Pharm Biopharm*, **103**, 109–117.
[105] Saboo, S., Tumban, E., Peabody, J., Wafula, D., Peabody, D.S., Chackerian, B. and Muttil, P. (2016) Optimized Formulation of a Thermostable Spray-Dried Virus-Like Particle Vaccine against Human Papillomavirus, *Molecular pharmaceutics*, **13**, 1646–1655.
[106] Bregu, M., Draper, S.J., Hill, A.V. and Greenwood, B.M. (2011) Accelerating vaccine development and deployment: report of a Royal Society satellite meeting, *Philos. Trans. R. Soc. London B Biol. Sci.*, **366**, 2841–2849.
[107] Trent, D.W. (2014) The regulatory path to vaccine, Vaccinology: An Essential Guide, Wiley, p. 212.
[108] Brennan, M.J., Fruth, U., Milstien, J. *et al*. (2007) Development of new tuberculosis vaccines: a global perspective on regulatory issues, *PLoS Med.*, **4**, e252.
[109] Tameris, M.D., Hatherill, M., Landry, B.S. *et al*. (2013) Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial, *Lancet*, **381**, 1021–1028.
[110] Garber, D.A., O'Mara, L.A., Zhao, J. *et al*. (2009) Expanding the repertoire of Modified Vaccinia Ankara-based vaccine vectors via genetic complementation strategies, *PLoS One*, **4**, e5445.
[111] Baldwin, S.L., Bertholet, S., Reese, V.A. *et al*. (2012) The importance of adjuvant formulation in the development of a tuberculosis vaccine, *J. Immunol.*, **188**, 2189–2197.
[112] Harrison, R.G., Todd, P.W., Rudge, S.R. and Petrides, D.P. (2015) Bioseparations science and engineering, Oxford University Press.
[113] Khan, A.S., Hughes, P. and Wiebe, W. (2010) Introduction to workshop: adventitious viruses in biologics - detection and mitigation strategies, *PDA J. Pharm. Sci. Technol.*, **65**, 544–546.
[114] Butel, J.S. (2000) Simian virus 40, poliovirus vaccines, and human cancer: research progress versus media and public interests, *Bull. World Health. Organ.*, **78**, 195–198.
[115] Gruber, M.F. (2012) Regulatory Pathways Supporting Development and Approval of Vaccines formulated with Novel Adjuvant: Regulatory Considerations and Challenges, Power Point Presentation, 1–30. http://www.fda.gov/downloads/EmergencyPreparedness/MedicalCountermeasures/UCM292045.pdf (accessed 11th March 2016).
[116] World Health Organization (2014) Assessing the programmatic suitability of vaccine candidates for WHO prequalification.
[117] World Health Organization (2005) Guidelines on the international packaging and shipping of vaccines.

5

TB Vaccine Assessment

Andre G. Loxton[a], Mary K. Hondalus[b] and Samantha L. Sampson[a]

[a] SA MRC Centre for TB Research, DST/NRF Centre of Excellence for Biomedical Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa
[b] Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, GA, United States

5.1 Introduction

The Stop TB partnership has stated a post-2015 target of a 90% reduction in tuberculosis (TB) incidence by 2035 [1]. New and effective drugs, diagnostic tools and vaccines will all be essential to achieving this goal. Of the three, vaccines represent one of the most cost-effective approaches to reducing morbidity and mortality associated with *Mycobacterium tuberculosis* infections. Currently, the only licensed vaccine available against TB is *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG), but this offers variable efficacy and does not confer life-long protection.

New vaccines currently in human trials to prevent *M. tuberculosis* disease are predominantly focused on prophylactic ability when administered to newborns or infants. More recently, post-exposure vaccines to aid in the prevention of reactivation or relapse of the active form of the disease are also being explored, along with therapeutic vaccines intended to be utilized as an adjunct to chemotherapeutic interventions. TB vaccine candidates currently in the global pipeline include live attenuated vaccines derived from mycobacteria, subunit vaccines consisting of selected mycobacterial antigens delivered either in viral vectors or with adjuvants, and mycobacterial lysates.

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

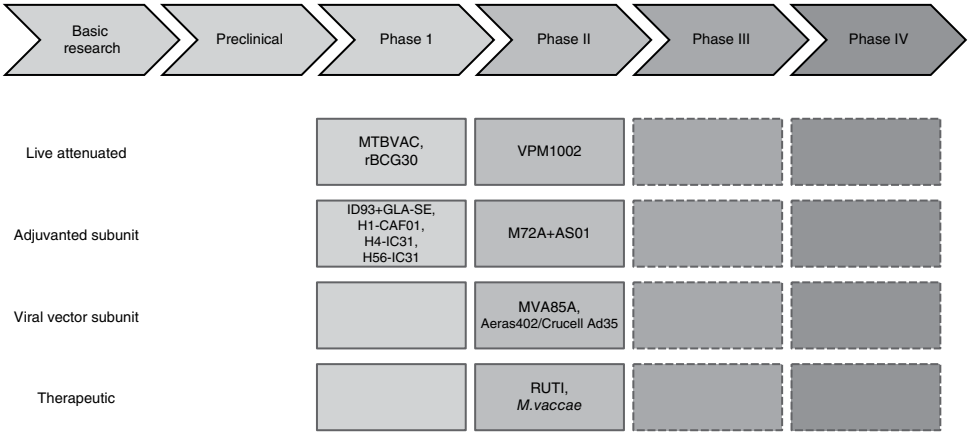


Figure 5.1 *Current status of TB vaccine Trial Pipeline. Categories of selected TB vaccine candidates discussed in this chapter, with examples at different stages of clinical trials (completed or ongoing). Dashed lines indicate trial phases/vaccine type combinations where no candidates have yet been tested*

This chapter provides an overview of the preclinical and clinical evaluation of selected TB vaccine candidates (Figure 5.1) with reference to animal models, human trials and laboratory immunological analysis to illustrate the pathways typical to TB vaccine development.

5.2 Preclinical Vaccine Assessment

Prior to human clinical trials, all vaccine candidates undergo rigorous safety, immunogenicity and efficacy testing. Initial safety testing could include *in vitro* assessments, for example formulation stability testing [2]. In the case of live attenuated vaccines, researchers might be required to demonstrate genetic stability [3]. These *in vitro* assessments are beyond the scope of the current chapter, and researchers undertaking these are strongly encouraged to work closely with local regulatory bodies to ensure that appropriate testing is conducted and documented [4, 5].

Following *in vitro* testing of new vaccine candidates, preclinical *in vivo* assessment will be carried out in multiple animal models. Regulatory approval will be dependent on positive outcomes from these models, which can provide essential information regarding safety, immunogenicity and protective efficacy. *In vivo* assessment of TB vaccine candidates is typically initiated in the murine model, which is the most accessible, tractable and cost-effective. Studies then generally progress to guinea pigs, followed by larger animals such as non-human primates and/or cattle. Details of different animal models utilized for TB vaccine assessment, in particular how well these recapitulate salient aspects of human disease, are provided in Chapter 3 of this volume and in several comprehensive reviews on the topic [6–8]. Importantly, all of these models show some improvement in their ability to control *M. tuberculosis* infection when immunized with *M. bovis* BCG, which is used as the gold standard in almost all preclinical vaccine studies.

The most advanced TB vaccine candidate to date is Modified Vaccinia virus Ankara expressing the immunodominant *M. tuberculosis* antigen Ag85A (MVA85A) (Oxford University) [9, 10]. MVA85A has been subjected to extensive preclinical testing in mice, guinea pigs, cattle and non-human primate models of human TB [7]. We will therefore use this candidate as an example to illustrate the types of assessments that can be undertaken in various animal models, as well as the advantages and limitations of the different models in the context of TB vaccine assessment.

5.2.1 Murine Model

As is typically the case for TB vaccine candidates, the first *in vivo* assessment of MVA85A was carried out in a murine model [11]. The model is widely accessible and benefits from the availability of numerous immunological reagents that allow the detailed characterization of responses to immunization and challenge. Mice are small and easily housed, facilitating the use of group sizes supporting studies that are sufficiently statistically powered. For certain types of vaccine candidates where the safety profile is a particular concern, for example live attenuated mycobacteria, initial studies may include severe combined immunodeficiency (SCID) mice [3]. This facilitates a rigorous assessment of safety in an immune-compromised host. However, the MVA vehicle already had substantial evidence of safety in human volunteers [12], and the first reported mouse study including MVA85A focused on immunogenicity and protective efficacy [11].

This initial study investigated intravenously administered MVA85A in combination with a DNA vaccine (also incorporating Ag85A) given via the intramuscular route [11]. Different prime-boost combinations of the two candidates were compared with BCG alone. In this study, mice were intraperitoneally challenged with 5–10 x 10^6 colony-forming units (CFU) *M. tuberculosis* H37Rv, relatively soon (2 weeks) after the last immunization. Readouts included interferon-gamma (IFN-γ) ELISPOT on splenocytes, as well as lung and spleen bacterial count determination. It should be noted that the route of immunization, high dose and unusual route of challenge, as well as the short interval between immunization and challenge used in this study are poorly reflective of the human clinical setting. Nonetheless, promising results were obtained, and a regimen incorporating 3 DNA immunizations followed by a single dose of MVA85A elicited the highest levels of IFN-γ-producing splenocytes out of the 4 combinations tested. This regimen also protected as well as did BCG in terms of reduction in lung organ burden compared with the non-immunized control group. These results therefore provided impetus for further development of the MVA85A candidate.

Two subsequent murine studies of MVA85A followed immunization and challenge routes more likely to reflect real-world deployment [13, 14]. These studies both utilized regimens with subcutaneous BCG priming and intradermal MVA85A boosting. In one of the studies, intranasal administration of both BCG and MVA85A was also investigated [13]. Challenge with *M. tuberculosis* H37Rv was with a lower dose (200–250 CFU) via the aerosol route, and took place 4 weeks after immunization. Both studies included readouts of bacterial organ burden, and IFN-γ ELISPOT assays. The later study also looked beyond IFN-γ, and used multi-parameter flow cytometry to enumerate multi-functional T-cell populations, as well as multiplex cytokine bead arrays [14]. Both of these studies reported a lack of correlation between systemic immunity and protection in the lung. Importantly though, upon intranasal immunization, protection in the lung correlated with levels of Ag85A-specific IFN-γ-secreting T-cells in lung lymph nodes [13]. Interestingly, intranasal

boosting with MVA85A elicited vaccine-induced protection better than did BCG alone, which very few TB vaccine candidates have achieved to date.

5.2.2 Guinea Pig Model

Guinea pigs are acutely susceptible to *M. tuberculosis* infection, and this host is therefore considered to offer a more stringent evaluation of vaccine efficacy than the murine model. The limited availability of immunological tools has presented historical impediments, but concerted efforts to develop reagents are overcoming this [15]. However, guinea pigs are more costly than mice and fewer laboratories have the capacity to house them under biosafety level 3 (BSL3) containment conditions. The limited number of laboratories able to support the model may be considered an advantage in some ways, as this encourages more head-to-head comparative evaluations of multiple vaccine candidates in a single experiment. This is essential for objective prioritization of candidates, where only a limited number can be progressed to human trials.

Assessment of MVA85A in guinea pigs has been carried out in the context of head-to-head evaluations intended to discriminate between vaccine candidates and to identify the highest priority candidates for further progression. Williams *et al*. tested 24 vaccine candidates, including MVA85A, in various regimens in 4 different experiments [16]. Candidates in this large comparative study were selected on the basis of previous data demonstrating immunogenicity and protective efficacy in mice (and in previous guinea pig studies for some candidates). Immunized animals were challenged with relatively high doses of *M. tuberculosis* H37Rv via the aerosol route, with the rationale that this would provide a stringent evaluation of vaccine efficacy to facilitate candidate prioritization. However, it should be considered that this may result in substantially different host responses to those elicited by natural, low-dose, infection, and may preclude the ability to discriminate between candidates. The 2 best performing regimens followed the BCG prime/viral vector boost design. Intradermal MVA85A boosting following subcutaneous BCG priming did not enhance protection afforded by BCG alone. An alternative regimen used subcutaneous BCG priming, followed by boosting with intradermal MVA85A, then a second boost with intradermal fowlpox-vectored Ag85A (FP85A). Boosting was performed at 4-week intervals, and the BCG-MVA85A-FP85A group was compared with BCG alone. Challenge with 500 CFU of *M. tuberculosis* H37Rv was performed via the aerosol route at 6 weeks after the last immunization. Protective efficacy was primarily assessed by survival over 17 or 26 weeks, and organ bacterial burden and histopathology was also assessed. The BCG-MVA85A-FP85A regimen was equivalent to BCG in terms of reduction of lung and spleen bacterial loads, and reduction in lung consolidation. Furthermore, this study highlighted the unprecedented finding that the BCG-MVA85A-FP85A regimen conferred 100% protection in terms of survival, out-performing BCG alone [16, 17]. However, it should be noted that the sample size used here was relatively small ($n=6$), and may not be statistically robust given the inherent biological variability in this model.

5.2.3 Cattle Model

Cattle have been extensively used to assess immunogenicity and protective efficacy of TB vaccine candidates [18, 19]. Bovine challenge experiments are very costly, with few sites worldwide capable of housing infected cattle in the required BSL3 containment

laboratories. However, this experimental system has yielded some important insights into the kinetics and nature of immune responses to vaccination and subsequent infection. An important feature of this model is that cattle are infected with *M. bovis*, thus representing a natural host–pathogen pairing. Not only is this a useful model for testing vaccines aimed at human populations, but bovine TB is an important target in its own right.

Two studies have reported the use of MVA85A in cattle. The first of these assessed the immunogenicity of different combinations of BCG, FP85A and MVA85A in prime-boost regimens [20]. Readouts included skin testing and assays on peripheral blood mononuclear cells (PBMCs) (IFN-γ ELISPOT, IFN-γ staining of $CD8^+$ T cells and the use of short-term T-cell lines to assess epitope specificity). This demonstrated that the inclusion of MVA85A in regimens with BCG significantly increased levels of Ag85A-specific IFN-γ-secreting T-cells. In addition, heterologous boosting broadened the resulting T-cell epitope repertoire.

A follow-up study investigated whether these promising results translated into enhanced protection against challenge [21]. In this study, FP85A was replaced with a different viral-vectored candidate, namely recombinant attenuated adenovirus expressing Ag85A (Ad85A). Cattle were subcutaneously primed with BCG, then intradermally boosted with either MVA85A or Ad85A after 8 weeks, and challenged with 2×10^3 CFU *M. bovis* via the intratracheal route 6 weeks following the boost. Non-vaccinated and BCG-vaccinated animals served as controls. Readouts included skin tests, pathology scores, enumeration of lymph node bacterial burden, whole-blood IFN-γ assay, IFN-γ ELISPOT, cultured ELISPOT and quantitative real-time polymerase chain reaction (RT-PCR) for selected cytokines. This study concluded that boosting BCG with the viral vectored candidates provided better protection than did BCG alone. The BCG prime/viral vector boost regimens elicited improvements in four of eight pathology parameters assessed, whereas BCG improved on only one parameter. The boosted regimens also reduced lymph node bacterial burdens, although this was only statistically significant for the Ad85A-boosted group. Grouping all vaccinated animals into "protected" and "non-protected" groups revealed possible predictors of vaccine efficacy. Interestingly, memory responses (as assessed by cultured ELISPOT assays) correlated with protection, as did Ag85A-induced IL-17 expression.

A potentially useful application of the cattle model is to assess transmission, and this has been explored by a number of researchers, using infected donor animals housed with sentinels [22–24]. Two studies conducted in such a natural transmission setting demonstrated that BCG immunization provided protection [22, 24]. However, more recent work has highlighted that inherent complexities of this model demand very large group sizes to provide sufficient statistically powered data [23]. The significant financial and logistical constraints involved would mean that such studies would be limited to only the most promising candidates. However, they may provide useful data regarding immune responses and correlates of protection in the context of natural infection.

5.2.4 Non-human Primate Model

Of all the animal models commonly in use for TB vaccine assessment, non-human primates are genetically, anatomically and physiologically most similar to humans. They are naturally susceptible to *M. tuberculosis* infection, and the disease manifestations closely

mirror that in humans. Importantly, the ability to co-infect animals with simian immunodeficiency virus (SIV) affords the ability to model the impact of immunization and subsequent challenge in HIV-positive humans [25, 26]. Although ethical and financial constraints mean that very few sites possess the capacity to perform TB vaccine testing in non-human primates, several TB vaccine candidates have been tested using this model.

Three separate studies (at two different study sites) have investigated safety, immunogenicity and protective efficacy of MVA85A in Rhesus macaques [27–29]. The first of these studies was reported in 2009 [28], at which time MVA85A was already undergoing clinical trials [30]. For some candidates, testing in non-human primates (in addition to small animal model testing) would be a regulatory requirement before human clinical trials would be allowed to proceed. However, the existing safety record of MVA85A allowed this step to be bypassed. Nonetheless, non-human primate studies were conducted in parallel to human testing to provide additional support for progression of this candidate.

The 3 reported non-human primate studies all utilized a BCG prime/MVA85A boost strategy [27–29], reflecting how the MVA85A candidate would likely be deployed in human populations. The first 2 studies utilized virtually identical immunization and challenge regimes (in terms of dose, route and timing), and both included challenge with a high dose (1000 CFU) of virulent *M. tuberculosis* Erdman via the respiratory route, 9 weeks after the last immunization [28]. The first study had a fixed-endpoint design, where all animals were euthanized at 16–17 weeks post-infection [28]. In contrast, the second study was conducted as a long-term survival study [27]. Both studies assessed pathology scores, clinical parameters (including weight) and organ bacterial burden. The fixed-endpoint study also assessed IFN-γ ELISA and chest X-rays [28], whereas the long-term survival study monitored IFN-γ ELISPOT results and performed magnetic resonance imaging (MRI) combined with stereology to determine lung lesion burden [27]. The fixed-endpoint study demonstrated that boosting with MVA85A protected at least as well as did BCG alone, and in fact the BCG prime/MVA85A boost regimen performed better than did BCG according to some parameters, including reduced lung bacterial burden [28]. Unfortunately, these promising results did not translate to enhanced long-term survival as measured in the second study. However, the authors noted that the MVA85A immunization in the second study did not significantly boost purified protein derivative (PPD)-specific responses in the BCG/MVA85A group. While the reason for this was unclear, it may explain the observed lack of protective efficacy. In support of this, the animal demonstrating the highest immune response following immunization with MVA85A showed the least severe disease symptoms and lowest organ burden at the end of the study. As highlighted by this study, because restricted group sizes (necessitated by financial and ethical factors) will limit statistical power, long-term survival studies offer little value in the non-human primate model. Fixed-endpoint designs are likely to be more useful.

The third, most recent, study of MVA85A in non-human primates was designed to compare the safety and immunogenicity of MVA85A delivered directly to the lungs to that associated with parenteral delivery [29]. All animals received a BCG prime, followed by an MVA85A boost via either the intradermal or respiratory route. Respiratory delivery of MVA85A exploited a nebulizer licensed for human use [29], making these results directly applicable to the clinical setting. This study detected no adverse effects attributable to the route of delivery. Both routes of MVA85A delivery elicited antigen-specific immune responses in the lung and periphery, with an apparent trend towards elevated responses in

the targeted compartment. Whether this translates into enhanced protection as has been observed for mucosal delivery of MVA85A in the murine model is unknown, but it does offer an exciting possibility for future study.

5.3 Clinical Assessment of Vaccines

5.3.1 Human Clinical Trials and Phases of Testing

Following preclinical testing of vaccines in various animal models, the safety, tolerability, immunogenicity and protective efficacy of the developed product need to be tested in humans. These clinical trials are performed over a number of years (up to 15 years), and progress through 4 phases of assessment.

Phase I trials are small, with only a few, normally healthy volunteers (up to about 50 people). Irrespective of the intended final target population, it is customary to perform Phase I testing of the vaccine in the country of origin. The primary aim of the trial is to check safety and tolerability of the vaccine with immunogenicity as a secondary or exploratory endpoint. Safety evaluations of TB vaccines can include blood chemistry tests (to monitor, for example, liver function and inflammatory responses), clinical observation of the participant (including injection-site evaluation for signs of swelling, pain, blistering) and self-reported symptoms (for example, malaise, headache). Another important feature of these trials is that they are aimed at finding the correct dose of vaccine to administer, so the small numbers of volunteers are even further divided into groups/cohorts. Investigators may also sample for assays aimed at understanding the *in vivo* mechanism of the vaccine in humans.

Phase II trials can include up to 100 or more participants. These trials now include the target population (e.g., newborns if the vaccine hopes to replace BCG) in a setting with a high incidence of the disease. This is done both to have sufficient numbers in a trial but also to compare with significant outcomes in the BCG group. Phase II also focuses on safety and efficacy, but with immunogenicity of the vaccine as a definite secondary outcome. The candidate vaccine, at the dose determined in Phase I, is then compared with BCG as given in the country of testing. For some trials there may also be a placebo group.

Phase III trials are large (more than 100 people, up to 1000) and can include multi-site and multi-country studies, in areas of high TB incidence. For these trials the focus is on protective efficacy and to further characterize the safety and immunogenicity profile. These trials are also randomized according to different algorithms and participants receive either vaccine or placebo. Outcomes of Phase III trials will determine whether the candidate will be approved and released to market.

Following vaccine approval and release to market, Phase IV trials investigate the long-term use of a product already classified as safe and efficacious.

5.3.2 Live Attenuated Vaccine Candidates

The prototypic live attenuated TB vaccine, BCG, was developed in the 1900s and first administered to humans in 1921 [31]. In excess of 3 billion doses of BCG have since been administered [32]. However, major differences in protective efficacy were observed in trials across the globe, ranging from over 70% protection in trials in the UK to 0% in India [33].

The failure of the available BCG vaccines to provide life-long protection led to the notion of improving the BCG vaccine. Approaches to improve BCG include the creation of recombinant strains that overexpress immunodominant mycobacterial antigens or heterologous proteins. Alternatively, BCG can be rationally mutated to enhance immunogenicity. One of the advantages of these strategies is that they build on BCG's extensive historical safety record, facilitating regulatory approval.

To date, two recombinant BCG strains have been assessed in human clinical trials. The first example, rBCG30, involved genetic modification of the BCG Tice strain by introduction of a plasmid to overexpress Antigen 85B (Ag85B). Following promising guinea pig studies [34], rBCG30 underwent a Phase I clinical trial. This vaccine significantly enhanced the frequency of Ag85B-specific CD4/CD8 T-cells, and produced a major increase in the number of antigen-specific T-cells able to inhibit intracellular mycobacterial replication [35].

Another recombinant BCG candidate vaccine, VPM1002 (Vakzine Projekt Management, GmbH), has advanced to Phase IIa clinical trials. VPM1002, alternatively known as *M. bovis* BCG Δ*ureC*::*hly*, exploits the dual strategies of a rationally targeted deletion and heterologous expression [36, 37]. VPM1002 overexpresses listeriolysin O, the virulence factor of *Listeria monocytogenes* encoded by the *hly* gene. In addition, the urease C gene has been deleted. The absence of urease C in VPM1002 compromises the ability of the strain to limit vacuole acidification, allowing for optimal functioning of listeriolysin O. Perforation of the phagosomal membrane by listeriolysin O allows outlet of recombinant antigens into the cytosol, which facilitates major histocompatibility complex (MHC)-mediated priming of CD8 T-cells. In murine experiments, VPM1002 vaccination elicited interleukin (IL)-17 production together with T-helper (Th) 1 cytokines. The produced IL-17 was able to enhance recruitment of Ag-specific T-cells to the lung [38].

Building on promising results in mice, guinea pigs, rabbits and non-human primates [37], clinical trials of VPM1002 were initiated. During the first clinical trial (Phase 1 open-labeled, dose escalation), healthy volunteers were randomized to receive one intradermal dose of BCG or VPM1002. Immunogenicity was evaluated in peripheral blood by assessing IFN-γ production, cellular immunity by flow cytometry, and antibodies against the mycobacterial antigens in the serum by enzyme-linked immunosorbent assay (ELISA) [36]. VPM1002 was found to be safe, well tolerated and immunogenic in terms of both T- and B-cell responses. In a completed Phase IIa study, further safety and immunogenicity of VPM1002 was evaluated in comparison with BCG in newborn infants (results pending, manuscript in preparation) (ClinicalTrials.gov ID: NCT01479972). This trial was sponsored by VPM, GmbH in partnership with Triclinium, the Children's Infectious Disease Research Unit (KidCru), and Stellenbosch University in South Africa.

While recombinant forms of BCG are showing promising results in animal and human studies, these formulations might pose inherent safety risks in immune-compromized individuals [39], and also lack a subset of antigens expressed by *M. tuberculosis* [40]. For these reasons, alternative approaches to generating live attenuated mycobacterial vaccines have been pursued. Several groups have developed live attenuated strains by rationally attenuating *M. tuberculosis* [41], reasoning that these would promote long-lived memory immune responses more closely resembling those elicited by natural infection. Safety concerns surrounding the use of live attenuated *M. tuberculosis* have been

addressed by extensive preclinical testing [3, 26, 42]. This has supported the approval of human Phase I clinical trials for one such candidate, MTBVAC. MTBVAC, developed by the University of Zaragoza, is a recombinant form of *M. tuberculosis* with two independent attenuating deletions [42]. This strain is deficient in the expression of several genes coding for virulence factors, including the Early Secretory Antigenic Target 6 kDa (ESAT6), and genes necessary for the synthesis of bacterial cell-wall components. In mouse and guinea pig studies, the MTBVAC vaccine was safe, well tolerated, immunogenic and protective against TB. MTBVAC was the first live attenuated vaccine based on *M. tuberculosis* to enter human clinical trial; a Phase I trial in Lausanne, Switzerland evaluated the safety and immunogenicity of MTBVAC compared with BCG in a dose-escalation study. The Phase I study was funded by Biofabri, S.L. in partnership with the University of Zaragoza, Tuberculosis Vaccine Initiative (TBVI), Centre Hospitalier Universitaire Vaudois, and the European Union Framework Partnership 7 (ClinicalTrials.gov Identifier: NCT02013245) [42].

5.3.3 Viral Vectored Subunit Vaccines

To date, the majority of subunit vaccine candidates have focused on a relatively small subset of immunodominant antigens selected on the basis of their ability to elicit robust T-cell responses, with a particular emphasis on the $CD4^+$ subset [6]. Interest has more recently expanded to include antigens thought to be important at different stages of disease, for example the so-called "latency antigens" [8]. Importantly, the antigen alone is unlikely to elicit a sufficiently robust immune response to confer protection, and current efforts are focused on delivery either with viral vectors or in adjuvanted formulations to enhance immunogenicity.

Currently one of the most advanced new TB vaccine candidates is MVA85A, a subunit viral vectored vaccine utilizing a recombinant strain of MVA to deliver antigen 85A of *M. tuberculosis* [9, 10]. Extensive preclinical testing as described above supported the progression of this candidate into human trials. The safety, tolerability and immunogenicity of the MVA85A vaccine is well documented through a number of clinical trials, which included healthy volunteers as well as infected children, infants and adults with *M. tuberculosis* and/or HIV [30, 43–48]. The vaccine has demonstrated the ability to induce antigen-specific polyfunctional CD4 T-cells as well as CD8 T-cells producing IFN-γ [44]. However, when tested in a Phase IIb trial in South Africa, boosting with the MVA85A vaccine did not confer any advantage above BCG alone, and was not able to protect healthy infants [10]. Another Phase II proof-of-concept, randomized, double-blind and placebo-controlled trial to assess the safety and immunogenicity profile of MVA85A against active tuberculosis disease in HIV-infected adults and healthy volunteers has been completed in South Africa and Senegal. Results indicate that even though the vaccine was immunogenic, it was not efficacious against *M. tuberculosis* infection or disease. However the study was not powered to detect vaccine-induced protection against disease [47] (ClinicalTrials.gov Identifier: NCT01151189). The developers of MVA85A are now conducting studies to evaluate the route of administration and correlating it to immune responses induced [49]. Oxford Emergent TB Consortium (OETC) and Aeras were the co-sponsors of the clinical development of the vaccine.

The Aeras 402/Crucell Ad35 vaccine is a replication-deficient adenovirus vector expressing the mycobacterial antigens TB10.4, Ag85A and Ag85B [50]. The vaccine elicited robust CD4 and CD8 T-cell responses when administered intranasally in a mouse model. In healthy BCG-vaccinated adults the vaccine had a tolerable safety profile; although vaccinees reported moderate local adverse events at the injection site, there were no reports of serious vaccine-related adverse events. The vaccine induced strong CD4 and CD8 T-cell responses, and CD4 T-cell responses were dominated by polyfunctional cells [51]. Results are pending for a small Phase I trial to evaluate the safety and immunogenicity profile of Aeras 402/Crucell Ad35 vaccine prime/MVA85A boost (ClinicalTrials.gov Identifier: NCT01683773), as well as a Phase II safety and immunogenicity trial in healthy infants (ClinicalTrials.gov Identifier: NCT01198366). Trials have been led and sponsored by Oxford University in partnership with Crucell Holland BV, Aeras, Emergent Biosolutions and the University of Birmingham.

5.3.4 Adjuvanted Subunit Vaccines

Several protein-adjuvant formulations are currently being assessed in clinical trials [52]. The most advanced of these is M72+AS01E (GlaxoSmithKline), which consists of two *M. tuberculosis* antigens, MTB32A (PPE18 protein), and MTB39A (a serine protease), formulated in the adjuvant AS01E. AS01E is a liposomally formulated adjuvant system containing the immunostimulants Monophosphoryl Lipid A (MPL) and *Quillaja saponaria* fraction 21 (QS21). The results of Phase I and Phase II clinical trials in different population groups and at different trial sites have been reported [53–57]. These demonstrate that M72+AS01E has an acceptable safety and reactogenicity profile (where reactogenicity refers to common transient adverse effects such as erythema, hematoma and swelling), including in infants [54] and in HIV-positive [57] and TB-positive adults [53, 56]. These studies also demonstrated the immunogenicity of the M72+AS01E formulation, as evidenced by both humoral and cell-mediated immune responses, the latter including the demonstration of polyfunctional M72-specific $CD4^+$ T-cell responses, even in HIV-infected adults [53–57]. A Phase IIb efficacy trial of M72+AS01E is currently ongoing in South Africa (ClinicalTrials.gov Identifier: NCT01755598). GlaxoSmithKline and Aeras co-sponsored the clinical development.

Hybrid 1 (H1) is a recombinant fusion protein of Ag85B and ESAT-6 developed by the Statens Serum Institute, which has been tested when adjuvanted with either IC31 or CAF01. The IC31 adjuvant system combines the immunostimulatory effects of an 11-mer antibacterial peptide, KLKL(5)KLK, and ODN1a, a synthetic oligodeoxynucleotide which is a Toll-like receptor 9 agonist [58]. The H1-IC31 vaccine candidate has progressed to Phase II clinical trials, and has been shown to be safe and immunogenic in both BCG-vaccinated and non-vaccinated individuals, *M. tuberculosis*-infected and uninfected individuals, as well as in HIV-infected adults [59–61]. IC31 has also been assessed in combination with two other recombinant fusion proteins in Phase I trials [52], but for the sake of brevity these will not be discussed here.

H1 has also been assessed in a formulation with CAF01, a two-component liposomal adjuvant, comprising the cationic liposome dimethyldioctadecylammonium (DDA) with a synthetic variant of "cord factor" (a mycobacterial cell-wall component) [62]. H1-CAF01 was safe and well tolerated in humans and produced durable T-cell immunity in healthy

non-BCG-vaccinated adults, characterized by double-positive IL-2/TNF-α-producing T-cells [63]. This, the first human study of CAF01, successfully demonstrated the ability of the adjuvant to elicit long-lasting Th1-focused cellular immunity, likely to be necessary for an effective TB vaccine.

A further protein-adjuvant formulation currently undergoing clinical trials is ID93 + GLA-SE (Infectious Diseases Research Institute, IDRI). ID93 is a fusion protein of four *M. tuberculosis* antigens, Rv2608 (PPE42), Rv3619 (EsxV), Rv3620 (EsxW), and Rv1813 (a latency-associated protein) [64]. GLA-SE is a glucopyranosyl lipid adjuvant formulated in squalene oil-in-water nanoemulsion, acting as a TLR4 agonist, shown to promote Th1-biased CD4+ responses [65]. ID93 + GLA-SE was well tolerated and protective in animal models of TB, and is currently undergoing Phase 1 clinical trials to evaluate its safety, tolerability, and immunogenicity in healthy adults (NCT01599897) and in BCG-vaccinated healthy adults (NCT01927159). Clinical development is co-sponsored by Aeras.

5.3.5 Therapeutic Vaccines

Several therapeutic vaccine candidates are currently under development [52]. One example, *Mycobacterium vaccae*, was initially developed as a therapeutic TB vaccine candidate, but trial results have been variable across different geographical settings [66]. No difference was observed between the treatment and placebo groups in a double-blind randomized controlled trial (RCT) in South Africa [67]. In patients with smear-positive TB a single dose of *M. vaccae* together with standard TB treatment in Malawi and Zambia was well tolerated [68]. The treatment had no influence on mortality or bacteriological results in the HIV-positive individuals, and only a tendency towards significant benefit in the HIV-negative cohort. Sputum-positive TB patients with no HIV infection in Uganda received a single dose [69] and this was comparable to a triple-dose regimen in Argentina as both were associated with faster radiological and bacteriological clearance compared with placebo. *M. vaccae* has since been assessed as a prophylactic vaccine [70].

RUTI® (Archivel Farma) is a therapeutic vaccine based upon detoxified liposomal cellular fragments of *M. tuberculosis* [71]. In a double-blind Phase I RCT in BCG-naïve healthy men in Spain, the vaccine was well tolerated and associated with modestly heightened responses to purified protein derivative and mycobacterial antigen including Ag85B and ESAT-6. RUTI vaccine is now expected to be evaluated in persons infected and diseased with *M. tuberculosis* [72].

5.3.6 Route of Immunization

To date the majority of vaccines are administered through injection, which has significant health and financial implications, and novel and effective, non-injectable delivery systems for vaccines are prioritized by the WHO. The importance is reinforced by the idea that mucosal vaccination in general, and intranasal vaccination specifically [73, 74], provides an improved immune response and protection compared with parenteral administration routes. Several groups are currently evaluating different mucosal vaccination options. For example, oral BCG vaccination of healthy adults previously vaccinated with BCG during childhood was well tolerated and meaningfully enhanced antigen-specific IFN-γ responses [75]. As mentioned below, the developers of MVA85A are also exploring mucosal delivery of this candidate [49].

5.4 Laboratory Immunological Analysis and Assessment of Vaccine Trials

5.4.1 Decision on Population of Interest

It is important to have access to a population with a known high incidence of TB; this provides a stringent test of vaccine potential and can help to accelerate trials. For instance, studies have demonstrated that the incidence of paediatric TB in some areas of the Western Cape Province in South Africa reaches 1–3% per annum [76, 77], subject to the definition of active disease used. This has supported the development of several clinical trial sites in this region, as has also been the case in other high-burden countries.

Optimal utilization of trial sites could be aided by stratification of populations according to genetic risk. The majority of people infected with *M. tuberculosis* present with latent TB infection and do not develop active TB disease [78], suggestive of natural resistance. Thirty to fifty percent of household contacts with substantial short-term exposure do not become infected [79], suggesting extensive heterogeneity in disease/infection susceptibility. Efforts to identify the genetic variants associated with pulmonary TB suggest that some individuals may have specific genetic risk factors associated with pulmonary TB [80]; this may support the grouping of trial volunteers most likely to develop disease to maximize statistical power.

5.4.2 Detection of Infection

If vaccines that prevent infection with *M. tuberculosis* are being sought, then it is important to be able to reliably detect infection. However, there is currently no gold standard immunological test for infection with *M. tuberculosis* and infection is indirectly assumed from measureable quantities of anti-mycobacterial immunity. The limitation is that these assays cannot distinguish a response to a new *M. tuberculosis* infection from that of a clinically latent (asymptomatic) infection with the bacterium. The relatively crude tuberculin skin test (TST) is the most commonly used method [81]. Two *in vitro* peripheral blood assays have been developed, which measure either the levels of IFN-γ-producing cells in response to *M. tuberculosis* antigens (IGRAs) or the secretion of IFN-γ by T-lymphocytes [82, 83]. Different aspects of anti-mycobacterial immunity are assessed by IGRAs and TSTs and these are not completely concordant in predicting infection with *M. tuberculosis*.

5.4.3 Detection of Protective Immunity

Protection against TB is predominantly T-cell immunity driven. The capacity to measure such immunity plays an important part in understanding the response to infection and disease. However, we lack a complete understanding of what constitutes protective immunity. Many studies have focused on measuring mycobacteria-specific IFN-γ responses, but it has become clear that while IFN-γ is necessary, it is not sufficient for protection against disease [6, 8]. More recent efforts have expanded analyses to enumerate polyfunctional T-cells, which were correlated with protection in animal models [84]. However, this has not held true in human studies. Efforts to identify reliable correlates of protection continue. Novel approaches to this problem include efforts to develop a human challenge model, where intradermal challenge with BCG is used to probe anti-mycobacterial immunity [85, 86]. This builds on proof-of-concept work in mice and cattle, where skin and intranodal

challenges, respectively, demonstrated that protection against the BCG challenge correlated with protection against challenge with *M. tuberculosis* [87, 88].

To further complicate matters, trials of several different types of vaccine candidates are being conducted at multiple trial sites, where differences in standard operating procedures can influence results obtained and complicate comparisons of different candidates, or even of the same candidate tested at different trial sites. Also important to note is that trial laboratories may wish to measure different features of the induced immune response (dependent on the vaccine candidate being tested) and this may lead to investigators opting for their assay of choice being employed. To some extent, this can be overcome by the harmonizing of assays and procedures [89], keeping in mind that the vaccines tested may differ with respect to their immunobiological mechanisms.

Important considerations include decisions on whether assays will be conducted with whole blood or peripheral blood mononuclear cells, and what the duration of antigenic stimulation will be (ranging from hours to several days). Another aspect of harmonizing protocols for vaccine immunology is the time delay before samples are processed. Several studies have demonstrated a decrease in immune response correlated with time before bloods are processed [90, 91]. It is now known that assays running for longer periods (e.g., proliferation assays running for 5–7 days, etc.) are less influenced by protocol variations, as these assays quantify growth of antigen-specific T-cells, and are thus less affected than are shorter-term assays that measure direct *ex vivo* function quantitatively [89].

To facilitate meaningful comparisons of different candidates and results from different trial sites, the WHO convened a panel of experts to identify challenges and to make recommendation with regards to immunological assays for TB vaccine trials. It is acknowledged that, in the absence of known immune correlates of risk and protection, a one-size-fits-all approach is unlikely to be suitable, and it is therefore recommended that all trials are evaluated with a range of immunological assays to capture as much information as possible related to the immune responses induced by the tested vaccines [89, 92]. It was also suggested that at least one simple, harmonized and standardized assay should be conducted in all trials, to allow direct comparisons of immunogenicity. The 7-day whole-blood IFN-γ assay was deemed suitable and has been standardized for use in different trial sites [89]. Even with the recommendation by the panel, this assay has not been implemented by all or reported on as being used during trials. Other challenges for TB vaccine trials include: the case definition of active TB disease, the cost and size of extended-scale trials, vaccine-testing sites and trained/skilled personnel as well as the ability of the producers of the vaccine to reduce the cost of the vaccine and distribution to as many countries as possible [93, 94]. These confounding factors need to be taken into account when preparing vaccines for testing and designing clinical trials.

5.5 How well do the Available Preclinical Models Predict Vaccine Success in Humans?

For MVA85A, the “failure” of the vaccine in Phase IIb clinical is perhaps not surprising when the animal model data are considered. While some studies did suggest improvement over BCG when MVA85A was included in vaccination regimens, these improvements

were small. In addition, the protective capacities of the strategies with the best outcomes in animal models (inclusion of FP85A or mucosal immunization with MVA85A) have not thus far been tested in humans.

When designing animal studies, it is important to reproduce the clinical setting as far as practically possible. An obvious factor to consider is the route of immunization. As described above, the majority of studies to date with MVA85A have been conducted using intradermal immunization, and indeed this has been the route of choice in the majority of clinical trials. However, the findings that mucosal boosting with MVA85A enhances protection over that conferred by BCG alone in mice [13], and elicits a trend toward elevated antigen-specific responses in targeted compartments in non-human primates [29], provide support for exploring this route of immunization in humans. Indeed, a Phase I clinical trial of aerosol delivery of MVA85A has provided promising immunological data, mirroring that found in non-human primates [49].

A potential criticism of many animal studies to date is that the timing of booster doses and subsequent challenge is usually not reflective of the clinical setting. However, financial and practical constraints limit the ability to design animal experiments of sufficient duration to address this. Apart from timing, there are other factors associated with the challenge component of animal studies that should be considered. The route of natural infection is via inhalation into the lung; this is reflected in most reported animal studies of the protective efficacy of TB vaccine candidates. However, the challenge dose used in almost all studies is much higher than what is expected to be encountered in natural infection; using very low challenge doses in a laboratory setting is usually not practically feasible, but it is nonetheless important to consider that high challenge doses represent a very stringent assessment of vaccine efficacy, and may trigger quite different immunological outcomes from natural infection. The challenge strain should also be considered. Inter-laboratory comparisons are obviously facilitated by the use of standardized laboratory strains such as *M. tuberculosis* H37Rv and *M. tuberculosis* Erdman, which have been used in the majority of studies. However, there is growing appreciation of strain-dependent disease outcomes [95], with some animal studies opting to include challenge with clinical isolates [64, 96, 97].

The TB vaccine field is in the fortunate position of having a number of promising vaccine candidates and several different animal models in which to test these. However, careful consideration needs to be given to how these models are utilized, and the benefits and limitations of each model should be weighed in light of the question being addressed. The cost and accessibility of each model plays an obvious role in decisions of which models to use at what stage of development. There is growing recognition of the importance of standardized, head-to-head comparisons of different vaccine candidates in preclinical models to ensure that the most promising candidates progress to human trials.

A major limitation of both human and animal studies is the lack of reliable correlates of risk and protection. However, the judicious use of animal models in conjunction with data emerging from ongoing clinical trials may help to address this. Animal models provide unique opportunities for detailed analysis of immunological and other parameters within specific compartments that are not always possible in humans. Integrating this information in an iterative fashion into our knowledge of human responses to immunization and infection may facilitate the identification of informative biosignatures, and ultimately underpin the licensing of new and effective TB vaccines.

References

[1] WHO (2013) Global strategy and targets for tuberculosis prevention, care and control after 2015.

[2] Wong, Y.L., Sampson, S., Germishuizen, W.A. *et al*. (2007) Drying a tuberculosis vaccine without freezing. *Proc. Natl. Acad. Sci. USA*, **104**(8), 2591–2595. PubMed PMID: 17299039. Pubmed Central PMCID: 1815227.

[3] Sampson, S.L., Dascher, C.C., Sambandamurthy, V.K. *et al*. (2004) Protection elicited by a double leucine and pantothenate auxotroph of Mycobacterium tuberculosis in guinea pigs. *Infect. Immun.*, **72**(5), 3031–3037. PubMed PMID: 15102816. Pubmed Central PMCID: 387862.

[4] Brennan, M.J. (2009) The US Food and Drug Administration provides a pathway for licensing vaccines for global diseases. *PLoS Med.*, **6**(7), e1000095. PubMed PMID: 19621065. Pubmed Central PMCID: 2705496.

[5] Brennan, M.J., Fruth, U., Milstien, J. *et al*. (2007) Development of new tuberculosis vaccines: a global perspective on regulatory issues. *PLoS Med.*, **4**(8), e252. PubMed PMID: 17683198. Pubmed Central PMCID: 1939860.

[6] Karp, C.L., Wilson, C.B. and Stuart, L.M. (2015) Tuberculosis vaccines: barriers and prospects on the quest for a transformative tool. *Immunol. Rev.*, **264**(1), 363–381. PubMed PMID: 25703572. Pubmed Central PMCID: 4368410.

[7] McShane, H. and Williams, A. (2014) A review of preclinical animal models utilised for TB vaccine evaluation in the context of recent human efficacy data. *Tuberculosis*, **94**(2), 105–110. PubMed PMID: 24369986. Pubmed Central PMCID: 3969587.

[8] Orme, I.M. (2011) Development of new vaccines and drugs for TB: limitations and potential strategic errors. *Future Microbiol.*, **6**(2), 161–177. PubMed PMID: 21366417. Pubmed Central PMCID: 3122326.

[9] Kaufmann, S.H., Lange, C., Rao, M. *et al*. (2014) Progress in tuberculosis vaccine development and host-directed therapies - a state of the art review. *Lancet Respir. Med.*, **2**(4), 301–320. PubMed PMID: 24717627.

[10] Tameris, M.D., Hatherill, M., Landry, B.S. *et al*. (2013) Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet*, **381**(9871), 1021–1028. PubMed PMID: 23391465.

[11] McShane, H., Behboudi, S., Goonetilleke, N. *et al*. (2002) Protective immunity against Mycobacterium tuberculosis induced by dendritic cells pulsed with both CD8(+)- and CD4(+)-T-cell epitopes from antigen 85A. *Infect. Immun.*, **70**(3), 1623–1626. PubMed PMID: 11854254. Pubmed Central PMCID: 127749.

[12] Gilbert, S.C. (2013) Clinical development of Modified Vaccinia virus Ankara vaccines. *Vaccine*, **31**(39), 4241–4246. PubMed PMID: 23523410.

[13] Goonetilleke, N.P., McShane, H., Hannan, C.M. *et al*. (2003) Enhanced immunogenicity and protective efficacy against Mycobacterium tuberculosis of bacille Calmette-Guerin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara. *J. Immunol.*, **171**(3), 1602–1609. PubMed PMID: 12874255.

[14] Tchilian, E.Z., Desel, C., Forbes, E.K. *et al*. (2009) Immunogenicity and protective efficacy of prime-boost regimens with recombinant (delta)ureC hly+ Mycobacterium bovis BCG and modified vaccinia virus ankara expressing M. tuberculosis antigen 85A against murine tuberculosis. *Infect. Immun.*, **77**(2), 622–631. PubMed PMID: 19064635. Pubmed Central PMCID: 2632018.

[15] Padilla-Carlin, D.J., McMurray, D.N. and Hickey, A.J. (2008) The guinea pig as a model of infectious diseases. *Comp. Med.*, **58**(4), 324–340. PubMed PMID: 18724774. Pubmed Central PMCID: 2706043.

[16] Williams, A., Hatch, G.J., Clark, S.O. *et a*l. (2005) Evaluation of vaccines in the EU TB Vaccine Cluster using a guinea pig aerosol infection model of tuberculosis. *Tuberculosis*, **85**(1–2), 29–38. PubMed PMID: 15687025.

[17] Williams, A., Goonetilleke, N.P., McShane, H. *et al*. (2005) Boosting with poxviruses enhances Mycobacterium bovis BCG efficacy against tuberculosis in guinea pigs. *Infect. Immun.*, **73**(6), 3814–3816. PubMed PMID: 15908420. Pubmed Central PMCID: 1111825.

[18] Buddle, B.M., Parlane, N.A., Wedlock, D.N. and Heiser, A. (2013) Overview of vaccination trials for control of tuberculosis in cattle, wildlife and humans. *Transbound. Emerg. Dis.*, **60** Suppl. 1, 136–146. PubMed PMID: 24171859.

[19] Waters, W.R., Palmer, M.V., Buddle, B.M. and Vordermeier, H.M. (2012) Bovine tuberculosis vaccine research: historical perspectives and recent advances. *Vaccine*, **30**(16), 2611–2622. PubMed PMID: 22342705.

[20] Vordermeier, H.M., Rhodes, S.G., Dean, G. *et al*. (2004) Cellular immune responses induced in cattle by heterologous prime-boost vaccination using recombinant viruses and bacille Calmette-Guerin. *Immunology*, **112**(3), 461–470. PubMed PMID: 15196215. Pubmed Central PMCID: 1782509.

[21] Vordermeier, H.M., Villarreal-Ramos, B., Cockle, P.J. *et al*. (2009) Viral booster vaccines improve Mycobacterium bovis BCG-induced protection against bovine tuberculosis. *Infect. Immun.*, **77**(8), 3364–3373. PubMed PMID: 19487476. Pubmed Central PMCID: 2715681.

[22] Ameni, G., Vordermeier, M., Aseffa, A. *et al*. (2010) Field evaluation of the efficacy of Mycobacterium bovis bacillus Calmette-Guerin against bovine tuberculosis in neonatal calves in Ethiopia. Clinical and vaccine immunology: *Clin. Vaccine Immunol.*, **17**(10), 1533–1538. PubMed PMID: 20719984. Pubmed Central PMCID: 2953002.

[23] Khatri, B.L., Coad, M., Clifford, D.J. *et al*. (2012) A natural-transmission model of bovine tuberculosis provides novel disease insights. *Vet. Rec.*, **171**(18), 448. PubMed PMID: 22935562.

[24] Lopez-Valencia, G., Renteria-Evangelista, T., Williams, Jde J. *et al*. (2010) Field evaluation of the protective efficacy of Mycobacterium bovis BCG vaccine against bovine tuberculosis. *Res. Vet. Sci.*, **88**(1), 44–49. PubMed PMID: 19564029.

[25] Jensen, K., Ranganathan, U.D., Van Rompay, K.K. *et al*. (2012) A recombinant attenuated Mycobacterium tuberculosis vaccine strain is safe in immunosuppressed simian immunodeficiency virus-infected infant macaques. *Clin. Vaccine Immunol.*, **19**(8), 1170–1181. PubMed PMID: 22695156. Pubmed Central PMCID: 3416096.

[26] Sampson, S.L., Mansfield, K.G., Carville, A. *et al*. (2011) Extended safety and efficacy studies of a live attenuated double leucine and pantothenate auxotroph of Mycobacterium tuberculosis as a vaccine candidate. *Vaccine*, **29**(29–30), 4839–4847. PubMed PMID: 21549795. Pubmed Central PMCID: 3146342.

[27] Sharpe, S.A., McShane, H., Dennis, M.J. *et al*. (2010) Establishment of an aerosol challenge model of tuberculosis in rhesus macaques and an evaluation of endpoints for vaccine testing. *Clin. Vaccine Immunol.*, **17**(8), 1170–1182. PubMed PMID: 20534795. Pubmed Central PMCID: 2916246.

[28] Verreck, F.A., Vervenne, R.A., Kondova, I. *et al*. (2009) MVA.85A boosting of BCG and an attenuated, phoP deficient M. tuberculosis vaccine both show protective efficacy against tuberculosis in rhesus macaques. *PLoS One*, **4**(4), e5264. PubMed PMID: 19367339. Pubmed Central PMCID: 2666807.

[29] White, A.D., Sibley, L., Dennis, M.J. *et al*. (2013) Evaluation of the safety and immunogenicity of a candidate tuberculosis vaccine, MVA85A, delivered by aerosol to the lungs of macaques. *Clin. Vaccine Immunol.*, **20**(5), 663–672. PubMed PMID: 23446219. Pubmed Central PMCID: 3647747.

[30] Beveridge, N.E., Price, D.A., Casazza, J.P. *et al*. (2007) Immunisation with BCG and recombinant MVA85A induces long-lasting, polyfunctional Mycobacterium tuberculosis-specific CD4+ memory T lymphocyte populations. *Eur. J. Immunol.*, **37**(11), 3089–3100. PubMed PMID: 17948267. Pubmed Central PMCID: 2365909.

[31] McShane, H. (2011) Tuberculosis vaccines: beyond bacille Calmette-Guerin. *Phil. Trans. R. Soc. London Ser. B, Biol. Sci.*, **366**(1579), 2782–2789. PubMed PMID: 21893541. Pubmed Central PMCID: 3146779.

[32] WHO (2013) BCG (Tuberculosis).

[33] Springett, V.H. and Sutherland, I. (1994) A re-examination of the variations in the efficacy of BCG vaccination against tuberculosis in clinical trials. *Tuberc. Lung Dis.*, **75**(3), 227–233. PubMed PMID: 7919317.

[34] Horwitz, M.A. and Harth, G. (2003) A new vaccine against tuberculosis affords greater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis. *Infect. Immun.*, **71**(4), 1672–1679. PubMed PMID: 12654780. Pubmed Central PMCID: 152073.

[35] Hoft, D.F., Blazevic, A., Abate, G. *et al*. (2008) A new recombinant bacille Calmette-Guerin vaccine safely induces significantly enhanced tuberculosis-specific immunity in human volunteers. *J. Infect. Dis.*, **198**(10), 1491–1501. PubMed PMID: 18808333. Pubmed Central PMCID: 2670060.

[36] Grode, L., Ganoza, C.A., Brohm, C. *et al*. (2013) Safety and immunogenicity of the recombinant BCG vaccine VPM1002 in a phase 1 open-label randomized clinical trial. *Vaccine*, **31**(9), 1340–1348. PubMed PMID: 23290835.

[37] Kaufmann, S.H., Cotton, M.F., Eisele, B. *et al*. (2014) The BCG replacement vaccine VPM1002: from drawing board to clinical trial. *Expert Rev. Vaccines*, **13**(5), 619–630. PubMed PMID: 24702486.

[38] Desel, C., Dorhoi, A., Bandermann, S. *et al*. (2011) Recombinant BCG DeltaureC hly+ induces superior protection over parental BCG by stimulating a balanced combination of type 1 and type 17 cytokine responses. *J. Infect. Dis.*, **204**(10),1573–1584. PubMed PMID: 21933877. Pubmed Central PMCID: 3192191.

[39] Hesseling, A.C., Cotton, M.F., Fordham von Reyn, C. *et al*. (2008) Consensus statement on the revised World Health Organization recommendations for BCG vaccination in HIV-infected infants. *Int. J. Tuberc. Lung Dis.*, **12**(12), 1376–1379. PubMed PMID: 19017445.

[40] Brusasca, P.N., Colangeli, R., Lyashchenko, K.P. *et al*. (2001) Immunological characterization of antigens encoded by the RD1 region of the Mycobacterium tuberculosis genome. *Scand. J. Immunol.*, **54**(5), 448–452. PubMed PMID: 11696195.

[41] Orme, I.M. (2013) Vaccine development for tuberculosis: current progress. *Drugs*, **73**(10), 1015–1024. PubMed PMID: 23794129. Pubmed Central PMCID: 3752905.

[42] Arbues, A., Aguil,o J.I., Gonzalo-Asensio, J. *et al*. (2013) Construction, characterization and preclinical evaluation of MTBVAC, the first live-attenuated M. tuberculosis-based vaccine to enter clinical trials. *Vaccine*, **31**(42), 4867–4873. PubMed PMID: 23965219.

[43] Dintwe, O.B., Day, C.L., Smit, E. *et al*. (2013) Heterologous vaccination against human tuberculosis modulates antigen-specific CD4+ T-cell function. *Eur. J. Immunol.*, **43**(9), 2409–2420. PubMed PMID: 23737382. Pubmed Central PMCID: 3816254.

[44] Hawkridge, T., Scriba, T.J., Gelderbloem, S. *et al*. (2008) Safety and immunogenicity of a new tuberculosis vaccine, MVA85A, in healthy adults in South Africa. *J. Infect. Dis.*, **198**(4), 544–552. PubMed PMID: 18582195. Pubmed Central PMCID: 2822902.

[45] Ibanga, H.B., Brookes, R.H., Hill, P.C. *et al*. (2006) Early clinical trials with a new tuberculosis vaccine, MVA85A, in tuberculosis-endemic countries: issues in study design. *Lancet Infect. Dis.*, **6**(8), 522–528. PubMed PMID: 16870530.

[46] Minassian, A.M., Rowland, R., Beveridge, N.E. *et al*. (2011) A Phase I study evaluating the safety and immunogenicity of MVA85A, a candidate TB vaccine, in HIV-infected adults. *BMJ Open*, **1**(2), e000223. PubMed PMID: 22102640. Pubmed Central PMCID: 3221299.

[47] Ndiaye, B.P., Thienemann, F., Ota, M. *et al*. (2015) Safety, immunogenicity, and efficacy of the candidate tuberculosis vaccine MVA85A in healthy adults infected with HIV-1: a randomised, placebo-controlled, phase 2 trial. *Lancet Respir. Med.*, **3**(3), 190–200. PubMed PMID: 25726088.

[48] Tameris, M., McShane, H., McClain, J.B. *et al*. (2013) Lessons learnt from the first efficacy trial of a new infant tuberculosis vaccine since BCG. *Tuberculosis*, **93**(2), 143–149. PubMed PMID: 23410889. Pubmed Central PMCID: 3608032.

[49] Satti, I., Meyer, J., Harris, S.A. *et al*. (2014) Safety and immunogenicity of a candidate tuberculosis vaccine MVA85A delivered by aerosol in BCG-vaccinated healthy adults: a phase 1, double-blind, randomised controlled trial. *Lancet Infect. Dis.*, **14**(10), 939–946. PubMed PMID: 25151225. Pubmed Central PMCID: 4178237.

[50] Hoft, D.F., Blazevic, A., Stanley, J. *et al*. (2012) A recombinant adenovirus expressing immunodominant TB antigens can significantly enhance BCG-induced human immunity. *Vaccine*, **30**(12), 2098–2108. PubMed PMID: 22296955.

[51] Abel, B., Tameris, M., Mansoor, N. *et al*. (2010) The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults. *Am. J. Resp. Crit. Care Med.*, **181**(12), 1407–1417. PubMed PMID: 20167847. Pubmed Central PMCID: 2894413.

[52] Weiner, J., 3rd and Kaufmann, S.H. (2014) Recent advances towards tuberculosis control: vaccines and biomarkers. *J. Intern. Med.*, **275**(5), 467-480. PubMed PMID: 24635488. Pubmed Central PMCID: 4238842.

[53] Day, C.L., Tameris, M., Mansoor, N. *et al*. (2013) Induction and regulation of T-cell immunity by the novel tuberculosis vaccine M72/AS01 in South African adults. *Am. J. Resp. Crit. Care Med.*, **188**(4), 492–502. PubMed PMID: 23306546. Pubmed Central PMCID: 3778736.

[54] Idoko, O.T., Owolabi, O.A., Owiafe, P.K. *et al*. (2014) Safety and immunogenicity of the M72/AS01 candidate tuberculosis vaccine when given as a booster to BCG in Gambian infants: an open-label randomized controlled trial. *Tuberculosis*, **94**(6), 564–578. PubMed PMID: 25305000.

[55] Leroux-Roels, I., Forgus, S., De Boever, F. *et al*. (2013) Improved CD4(+) T cell responses to Mycobacterium tuberculosis in PPD-negative adults by M72/AS01 as compared to the M72/AS02 and Mtb72F/AS02 tuberculosis candidate vaccine formulations: a randomized trial. *Vaccine*, **31**(17), 2196–2206. PubMed PMID: 22643213.

[56] Montoya, J., Solon, J.A., Cunanan, S.R. *et al*. (2013) A randomized, controlled dose-finding Phase II study of the M72/AS01 candidate tuberculosis vaccine in healthy PPD-positive adults. *J. Clin. Immunol.*, **33**(8), 1360–1375. PubMed PMID: 24142232. Pubmed Central PMCID: 3825318.

[57] Thacher, E.G., Cavassini, M., Audran, R. *et al*. (2014) Safety and immunogenicity of the M72/AS01 candidate tuberculosis vaccine in HIV-infected adults on combination antiretroviral therapy: a phase I/II, randomized trial. *Aids*, **28**(12), 1769–1781. PubMed PMID: 24911353.

[58] Schellack, C., Prinz, K., Egyed, A. *et al*. (2006) IC31, a novel adjuvant signaling via TLR9, induces potent cellular and humoral immune responses. *Vaccine*, **24**(26), 5461-5472. PubMed PMID: 16678312.

[59] Reither, K., Katsoulis, L., Beattie, T. *et al*. (2014) Safety and immunogenicity of H1/IC31(R), an adjuvanted TB subunit vaccine, in HIV-infected adults with CD4+ lymphocyte counts greater than 350 cells/mm3: a phase II, multi-centre, double-blind, randomized, placebo-controlled trial. *PLoS One*, **9**(12), e114602. PubMed PMID: 25490675. Pubmed Central PMCID: 4260867.

[60] van Dissel, J.T., Arend, S.M., Prins, C. *et al*. (2010) Ag85B-ESAT-6 adjuvanted with IC31 promotes strong and long-lived Mycobacterium tuberculosis specific T cell responses in naive human volunteers. *Vaccine*, **28**(20), 3571–3581. PubMed PMID: 20226890.

[61] van Dissel, J.T., Soonawala, D., Joosten, S.A. *et al*. (2011) Ag85B-ESAT-6 adjuvanted with IC31(R) promotes strong and long-lived Mycobacterium tuberculosis specific T cell responses in volunteers with previous BCG vaccination or tuberculosis infection. *Vaccine*, **29**(11), 2100–2109. PubMed PMID: 21256189.

[62] Christensen, D., Foged, C., Rosenkrands, I. *et al*. (2010) CAF01 liposomes as a mucosal vaccine adjuvant: In vitro and in vivo investigations. *Int. J. Pharm.*, **390**(1), 19–24. PubMed PMID: 19879346.

[63] van Dissel, J.T., Joosten, S.A., Hoff, S.T. *et al*. (2014) A novel liposomal adjuvant system, CAF01, promotes long-lived Mycobacterium tuberculosis-specific T-cell responses in human. *Vaccine*, **32**(52), 7098–7107. PubMed PMID: 25454872.

[64] Bertholet, S., Ireton, G.C., Ordway, D.J. *et al*. (2010) A defined tuberculosis vaccine candidate boosts BCG and protects against multidrug-resistant Mycobacterium tuberculosis. *Sci. Transl. Med.*, **2**(53), 53–74. PubMed PMID: 20944089. Pubmed Central PMCID: 3110937.

[65] Baldwin, S.L., Bertholet, S., Reese, V.A. *et al*. (2012) The importance of adjuvant formulation in the development of a tuberculosis vaccine. *J. Immunol.*, **188**(5), 2189–2197. PubMed PMID: 22291184. Pubmed Central PMCID: 3288309.

[66] Dlugovitzky, D., Fiorenza, G., Farroni, M. *et al*. (2006) Immunological consequences of three doses of heat-killed Mycobacterium vaccae in the immunotherapy of tuberculosis. *Resp. Med.*, **100**(6), 1079–1087. PubMed PMID: 16278080.

[67] Mayo, R.E. and Stanford, J.L. (2000) Double-blind placebo-controlled trial of Mycobacterium vaccae immunotherapy for tuberculosis in KwaZulu, South Africa, 1991-97. *Trans. R. Soc. Trop. Med. Hyg.*, **94**(5), 563–568. PubMed PMID: 11132390.

[68] Crampin, A.C., Glynn, J.R. and Fine, P.E. (2009) What has Karonga taught us? Tuberculosis studied over three decades. *Int. J. Tuberc. Lung Dis.*, **3**(2), 153–164. PubMed PMID: 19146741. Pubmed Central PMCID: 3272402.

[69] Johnson, J.L., Kamya, R.M., Okwera, A. *et al*. (2000) Randomized controlled trial of Mycobacterium vaccae immunotherapy in non-human immunodeficiency virus-infected ugandan adults with newly diagnosed pulmonary tuberculosis. The Uganda-Case Western Reserve University Research Collaboration. *J. Infect. Dis.*, **181**(4), 1304–1312. PubMed PMID: 10753731.

[70] Dlugovitzky, D., Notario, R., Martinel-Lamas, D. *et al*. (2010) Immunotherapy with oral, heat-killed, Mycobacterium vaccae in patients with moderate to advanced pulmonary tuberculosis. *Immunotherapy*, **2**(2), 159–169. PubMed PMID: 20635925.

[71] Cardona, P.J. (2006) RUTI: a new chance to shorten the treatment of latent tuberculosis infection. *Tuberculosis*, **86**(3–4), 273–289. PubMed PMID: 16545981.

[72] Vilaplana, C., Montane, E., Pinto, S. *et al*. (2010) Double-blind, randomized, placebo-controlled Phase I Clinical Trial of the therapeutical antituberculous vaccine RUTI. *Vaccine*, **28**(4), 1106–1116. PubMed PMID: 19853680.

[73] Manjaly Thomas, Z.R. and McShane, H. (2015) Aerosol immunisation for TB: matching route of vaccination to route of infection. *Trans. R. Soc. Trop. Med. Hyg.*, **109**(3), 175–181. PubMed PMID: 25636950. Pubmed Central PMCID: 4321022.

[74] Wang, J., Thorson, L., Stokes, R.W. *et al*. (2004) Single mucosal, but not parenteral, immunization with recombinant adenoviral-based vaccine provides potent protection from pulmonary tuberculosis. *J. Immunol.*, **173**(10), 6357–6365. PubMed PMID: 15528375.

[75] Monteiro-Maia, R., Ortigao-de-Sampaio, M.B., Pinho, R.T. and Castello-Branco, L.R. (2006) Modulation of humoral immune response to oral BCG vaccination by Mycobacterium bovis BCG Moreau Rio de Janeiro (RDJ) in healthy adults. *J. Immune Based Ther. Vaccines*, **4**, 4. PubMed PMID: 16956404. Pubmed Central PMCID: 1569376.

[76] Claassens, M., van Schalkwyk, C., den Haan, L. *et al*. (2013) High prevalence of tuberculosis and insufficient case detection in two communities in the Western Cape, South Africa. *PLoS One*, **8**(4), e58689. PubMed PMID: 23560039. Pubmed Central PMCID: 3613399.

[77] Soeters, M., de Vries, A.M., Kimpen, J.L. *et al*. (2005) Clinical features and outcome in children admitted to a TB hospital in the Western Cape - the influence of HIV infection and drug resistance. *S.Afr.Med.J. = Suid-Afrikaanse Tydskrif vir Geneeskunde*, **95**(8), 602–606. PubMed PMID: 16201005.

[78] Fine, P.E., Sterne, J.A., Ponnighaus, J.M. and Rees, R.J. (1994) Delayed-type hypersensitivity, mycobacterial vaccines and protective immunity. *Lancet*, **344**(8932), 1245–1249. PubMed PMID: 7967984.

[79] Marks, S.M., Taylor, Z., Qualls, N.L. *et al*. (2000) Outcomes of contact investigations of infectious tuberculosis patients. *Am. J. Resp. Crit. Care Med.*, **162**(6), 2033–2038. PubMed PMID: 11112109.

[80] Moller, M. and Hoal, E.G. (2010) Current findings, challenges and novel approaches in human genetic susceptibility to tuberculosis. *Tuberculosis*, **90**(2), 71–83. PubMed PMID: 20206579.

[81] Machado, A., Jr., Emodi, K., Takenami, I. *et al*. (2009) Analysis of discordance between the tuberculin skin test and the interferon-gamma release assay. *Int. J. Tuberc. Lung Dis.*, **13**(4), 446–453. PubMed PMID: 19335949.

[82] Adetifa, I.M., Lugos, M.D., Hammond, A. *et al*. (2007) Comparison of two interferon gamma release assays in the diagnosis of Mycobacterium tuberculosis infection and disease in The Gambia. *BMC Infect. Dis.*, **7**, 122. PubMed PMID: 17961228. Pubmed Central PMCID: 2216027.

[83] Jung, Y.J., Lyu, J., Yoo, B. *et al*. (2012) Combined use of a TST and the T-SPOT(R).TB assay for latent tuberculosis infection diagnosis before anti-TNF-alpha treatment. *Int. J. Tuberc. Lung Dis.*, **16**(10), 1300–1306. PubMed PMID: 22863375.

[84] Forbes, E.K., Sander, C., Ronan, E.O. *et al*. (2008) Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against Mycobacterium tuberculosis aerosol challenge in mice. *J. Immunol.*, **181**(7), 4955–4964. PubMed PMID: 18802099. Pubmed Central PMCID: 2867031.

[85] Harris, S.A., Meyer, J., Satti, I. *et al*. (2014) Evaluation of a human BCG challenge model to assess antimycobacterial immunity induced by BCG and a candidate tuberculosis vaccine, MVA85A, alone and in combination. *J. Infect. Dis.*, **209**(8), 1259–1268. PubMed PMID: 24273174. Pubmed Central PMCID: 3969545.

[86] Minassian, A.M., Satti, I., Poulton, I.D. *et al*. (2012) A human challenge model for Mycobacterium tuberculosis using Mycobacterium bovis bacille Calmette-Guerin. *J. Infect. Dis.*, **205**(7), 1035–1042. PubMed PMID: 22396610. Pubmed Central PMCID: 3295601.

[87] Minassian, A.M., Ronan, E.O., Poyntz, H. *et al*. (2010) Preclinical development of an in vivo BCG challenge model for testing candidate TB vaccine efficacy. *PLoS One*, **6**(5), e19840. PubMed PMID: 21629699. Pubmed Central PMCID: 3101220.

[88] Villarreal-Ramos, B., Berg, S., Chamberlain, L. *et al*. (2014) Development of a BCG challenge model for the testing of vaccine candidates against tuberculosis in cattle. *Vaccine*, **32**(43), 5645–5649. PubMed PMID: 25138291.

[89] Hanekom, W.A., Dockrell, H.M., Ottenhoff, T.H. *et al*. (2008) Immunological outcomes of new tuberculosis vaccine trials: WHO panel recommendations. *PLoS Med.*, **5**(7), e145. PubMed PMID: 18597551. Pubmed Central PMCID: 2443198.

[90] Blimkie, D., Fortuno, E.S., 3rd, Yan, H. *et al*. (2011) Variables to be controlled in the assessment of blood innate immune responses to Toll-like receptor stimulation. *J. Immunol. Methods*, **366**(1–2), 89–99. PubMed PMID: 21277305. Pubmed Central PMCID: 3051022.

[91] Naranbhai, V., Bartman, P., Ndlovu, D. *et al*. (2011) Impact of blood processing variations on natural killer cell frequency, activation, chemokine receptor expression and function. *J. Immunol. Methods*, **366**(1–2), 28–35. PubMed PMID: 21255578. Pubmed Central PMCID: 3051017.

[92] Hoft, D.F. (2008) Tuberculosis vaccine development: goals, immunological design, and evaluation. *Lancet*, **372**(9633), 164–175. PubMed PMID: 18620952.

[93] Brennan, M.J. (2005) The tuberculosis vaccine challenge. *Tuberculosis*, **85**(1–2), 7–12. PubMed PMID: 15687021.

[94] Checkley, A.M. and McShane, H. (2011) Tuberculosis vaccines: progress and challenges. *Trends Pharmacol. Sci.*, **32**(10), 601–606. PubMed PMID: 21803435.

[95] Kato-Maeda, M., Shanley, C.A., Ackart, D. *et al*. (2012) Beijing sublineages of Mycobacterium tuberculosis differ in pathogenicity in the guinea pig. *Clin. Vaccine Immunol.*, **19**(8), 1227–1237. PubMed PMID: 22718126. Pubmed Central PMCID: 3416080.

[96] Grode, L., Seiler, P., Baumann, S. *et al*. (2005) Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guerin mutants that secrete listeriolysin. *J. Clin. Invest.*, **115**(9), 2472–2479. PubMed PMID: 16110326. Pubmed Central PMCID: 1187936.

[97] Ordway, D.J., Shang, S., Henao-Tamayo, M. *et al*. (2011) Mycobacterium bovis BCG-mediated protection against W-Beijing strains of Mycobacterium tuberculosis is diminished concomitant with the emergence of regulatory T cells. *Clin. Vaccine Immunol.*, **18**(9), 1527–1535. PubMed PMID: 21795460. Pubmed Central PMCID: 3165219.

Section 3
Drug Treatment

6

Testing Inhaled Drug Therapies for Treating Tuberculosis

Ellen F. Young[a], *Anthony J. Hickey*[b] *and Miriam Braunstein*[a]

[a] *Department of Microbiology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, NC, USA*

[b] *Technology for Industry and the Environment, Discovery Sciences Technologies Group, RTI International, Research Triangle Park, NC, USA*

6.1 Introduction

The world desperately needs new and improved drugs to treat tuberculosis (TB). On top of an increasing drug resistance problem, current therapies are long and have side effects. Inhaled therapies have revolutionized the treatment of asthma and other pulmonary diseases. A small number of studies indicate the same approach could be a beneficial alternative or addition to current TB treatment. In this Chapter, we review examples of inhaled therapies in current practice and animal studies that indicate the promise of this approach for treating TB. We then review specifics of how to perform inhaled drug efficacy studies using the guinea pig model of TB and aerosol dosing chambers. We hope this Chapter will serve as a practical guide for researchers who want to test the efficacy of TB drugs delivered by inhalation and inspire others to consider doing so.

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

6.2 The Need for New Drug Treatments for Tuberculosis

The introduction of antibiotics to treat tuberculosis (TB) in the 1940s held great promise for eradication of this infamous disease. However, our current need for new and more effective treatments for TB could not be greater or more urgent. Current drug treatment for active TB is six months long and requires high doses of four orally delivered antibiotics: rifampicin, isoniazid, pyrazinamide and ethambutol. For TB patients with visible cavities in their lungs, the treatment is extended to 9 months [1]. The lengthy treatment period combined with side effects of this drug regimen negatively impact patient adherence to treatment, and this has contributed to the development of drug-resistant strains. Multidrug-resistant, extensively drug-resistant and totally drug-resistant TB cases (MDR, XDR, and TDR) are increasing each year. MDR TB is defined as being resistant to rifampicin and isoniazid [2]. XDR is an MDR strain that is additionally resistant to any fluoroquinolone (such as ofloxacin or moxifloxacin) and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin). TDR, the most frightening of all, is TB that is currently untreatable. In 2013, there were 9 million new cases of TB including 480,000 cases of MDR TB, of which 9% were thought to be XDR [2]. For drug-resistant TB, treatment is much more expensive, longer (~2 years), and it requires extremely potent antibiotics with serious side effects (e.g., depression, psychosis, hearing loss, hepatitis, and kidney impairment). Some of the drugs available for MDR and XDR TB are only available as injectables, making treatment even more onerous [3]. With MDR and XDR TB, treatment fatigue and lack of adherence is an even bigger problem than is associated with the standard four-drug therapy for drug-sensitive TB [4]. The TB crisis is additionally confounded by the high number of HIV cases in areas of the world where TB is most prevalent. HIV increases the likelihood of active transmissible TB infection, either through new infection or reactivation of latent TB. The combination of HIV and drug-resistant TB in the same individual is not only devastating for the individual but it drives further dissemination of drug-resistant TB. Given the current problems facing TB treatment, novel and shorter therapies desperately need to be developed.

6.3 Inhaled Drug Therapy for Tuberculosis

Currently, TB drugs are delivered either orally or by injection. However, for a pulmonary disease like TB the potential of treating with inhaled drugs holds great appeal [5–7]. In recent years, inhaled drug therapies have proven stable, easy to deliver and effective. For asthma, inhaled therapies are now common. Better disease control and fewer side effects are associated with aerosol delivery of the same asthma drugs that were given orally for decades [8]. Other inhaled therapies in current practice are FDA-approved aerosolized antibiotics (tobramycin, aztreonam and colistimethate) for chronic *Pseudomonas aeruginosa* lung infections in cystic fibrosis patients [9, 10]. Inhaled drug therapy is also used to deliver pentamidine to treat *Pneumocystis carinii* pneumonia [11]. Finally, another example is amphotericin B, which is used to prevent fungal infection in lung-transplant patients. Toxic side effects associated with amphotericin B delivered as an injectable drug are overcome when it is delivered as a nebulized aerosol at lower doses [12, 13]. Given the need to explore all therapeutic possibilities for TB treatment, the efficacy of inhaled drug therapy

deserves consideration and evaluation. Interestingly, back in 1950, aerosolized streptomycin was briefly used to successfully treat TB [14]. However, there are currently no approved inhaled therapies for TB.

For TB, inhalation therapy would have the benefit of targeted delivery of high drug concentrations directly to the site of infection. Higher drug levels in the lungs may reduce the time of current therapies, which would improve patient adherence. Further, targeted lung delivery could be especially useful for treating latent TB, in which the *Mycobacterium tuberculosis* bacteria are encased in multicellular, poorly vascularized, and calcified granulomas that hinder drug penetration with standard oral therapy. Following oral administration, drugs must traverse the gut, enter the blood stream, and travel to the site of infection in the lungs. There are diminishing concentrations of drug at each step of the process. An excellent discussion of oral drug distribution and penetration into lung granulomas can be found in the review of V. Dartois, in Nature Reviews Microbiology, 2014 [15]. By avoiding the pH extremes of the stomach/intestines and the need for drug adsorption from the gut, inhalation therapy is a particularly attractive option for delivering acid-labile or poorly adsorbed drugs for TB. In addition, some drugs have solubility problems that make them difficult to administer orally or by injection but, when formulated as dry powders, they can be delivered by inhalation. Finally, a benefit of inhaled therapy is that high systemic concentrations can be largely avoided, which reduces the potential for toxic side effects as occurs with several TB drugs. Adherence to inhalation therapy is typically very good and this is most likely due to reduced side effects.

Not only does aerosol delivery of TB drugs have the potential to achieve higher local drug concentrations that can lead to shorter time to cure, it also has the possibility of shortening the time that an infected individual can spread TB to others. TB is transmitted by respiratory droplets of *M. tuberculosis* released from the airway by coughing, sneezing or talking. By directly delivering drugs to the airway of infected individuals, the length of time that a person can transmit TB bacteria will diminish more rapidly.

6.4 Published Studies of Inhalation Therapy for TB

While the number of studies testing the efficacy of aerosol-delivered TB drugs remains small, experiments show this approach to be a promising alternative or addition to standard treatment with first-line drugs. In the very first study of this type, a single dose of rifampicin was delivered by insufflation to the lungs as particles encased in slow-releasing polylactide-co-glycolide (PLG) microspheres. In this study, rifampicin particles were delivered prior to infection of guinea pigs with *M. tuberculosis* to test efficacy prophylactically. While the treatment did not completely prevent infection, it was effective in significantly reducing the *M. tuberculosis* burden [measured as colony-forming units (CFU)] in the lungs [16]. In a subsequent study, daily treatment with nebulized rifampicin delivered as microparticles of free drug was tested following establishment of TB infection. This treatment reduced the bacterial burden in the lungs and spleen compared with untreated guinea pigs [17]. When combination therapy of rifampicin, isoniazid and pyrazinamide (RHZ) was delivered to the lungs of TB-infected guinea pigs as free-drug or in PLG or lectin-functionalized PLG microspheres, a 4 log reduction of CFU was achieved in the lungs and spleens after 4–5 doses delivered over a 45-day period [18, 19]. In another study, RHZ

loaded into solid lipid particles reduced the lung and spleen CFU burden by more than 4 logs after 7 nebulized doses to TB infected guinea pigs [20].

In addition to considering inhaled delivery of first-line TB drugs, many second-line anti-TB drugs are particularly attractive candidates for pulmonary delivery due to their incompatibility with oral dosing and requirement for intravenous or intramuscular injection. Capreomycin is a second-line drug that is typically given daily for 60–120 days by intramuscular injection and is associated with significant side effects. Aerosol treatment with capreomycin, formulated into microparticles containing 80% capreomycin and 20% leucine, resulted in a significant 1 log decrease in lung CFU of TB-infected guinea pigs. This reduction in lung CFU was comparable to what was achieved with 30% higher drug levels delivered to guinea pigs by intramuscular injection in parallel [21]. Spectinamides, which are a promising new class of anti-TB drug, have been delivered to the lungs of TB-infected mice by intratracheal insufflation and have been shown to be effective also [22]. Finally, gentamicin is a drug with anti-TB activity that is not currently used because the levels required to be effective are not attainable systemically. TB-infected mice exhibited a 1.5 log decrease in lung CFU after 4 weeks of daily treatment with aerosolized gentamicin [23]. In 2001, inhaled gentamicin or kanamycin, the latter being another aminoglycoside with anti-mycobacterial activity, was given to 19 people with unusually persistent positive *M. tuberculosis* sputum smears. Thirteen of the individuals treated converted to negative smears with a median time of 23 days, and no recurrence of disease was observed [24]. Thus, animal and human studies performed to date support the potential of using inhaled therapies to improve current TB treatment by making it more efficacious and patient friendly.

6.5 The Guinea Pig Model for Testing Inhaled Therapies for TB

Guinea pigs and mice can be used to test aerosolized drugs for their effectiveness in treating TB. While rabbits and nonhuman primates are excellent models for TB, there have been no reports of inhaled TB drugs being tested in either model. Nonhuman primates, however, have been used in aerosolized vaccine trials [25] and could be similarly used to test inhaled TB drug efficacy, although the monetary costs of this model are high. Mice are the most cost effective animal model for TB studies and there are numerous tools for studying physiology and immune responses in the mouse as well as for testing the fate of aerosol particles [26]. However, there are disadvantages for using mice as a model for human TB and specifically for testing the efficacy of inhaled therapies, which has led to the guinea pig being the small-animal model of choice for these studies [27]. Among the differences between TB infection of mice and humans are that mice require 50–100 aerosolized bacteria to be reproducibly infected with *M. tuberculosis*, as opposed to humans that can be infected with as few as 1–10 aerosolized *M. tuberculosis* bacteria [28]. In addition, the cellular granulomas that form in mice to contain *M. tuberculosis* resemble early-stage human granulomas but they do not progress further to a structure resembling late-stage human granulomas [29, 30]. Murine granulomas are less organized, they do not become hypoxic and the caseum does not liquefy in comparison with human granulomas. Since drug penetration into hypoxic granulomas is a formidable problem in the treatment of TB, the different features of murine granulomas is a drawback for using the mouse model for testing drugs. It is

noteworthy, however, that C3HeB/FeJ (Kramnik) mice form necrotic granulomas that are better developed [31], and these mice are currently being evaluated for use in drug testing [32]. Additionally, mice infected with TB do not develop a delayed hypersensitivity reaction to TB proteins (purified protein derivative; PPD), which is another difference between mice and humans [27].

In comparison, the course of *M. tuberculosis* infection in guinea pigs is more similar to what is seen in humans. Guinea pigs are susceptible to low-dose aerosol infection (1–10 bacteria), and they develop hypoxic caseous granulomas with necrotic centers, which are the hallmark for progressive TB in humans [29, 30]. Guinea pigs also have a delayed hypersensitivity reaction to a PPD skin test after TB exposure or vaccination with the live attenuated BCG vaccine. The similarities between guinea pig and human TB disease may explain why some results obtained with guinea pigs (for example, moxifloxacin substitution for isoniazid in TB treatment) have proven more predictive of results in humans than have studies performed in mice [33].

For specifically testing inhaled drugs, mice present additional drawbacks compared with guinea pigs. In mice, the pathway an aerosol must follow to reach the lungs is longer and more circuitous than in guinea pigs or humans. In addition, mice have small alveoli and airways, which results in a small tidal volume (volume of a single breath). Consequently, very long dosing times are required to deliver drugs by inhalation to mice. Therefore, when pulmonary delivered drugs are tested in mice, insufflation or instillation is generally used. During insufflation a long tube is inserted into the trachea and the dispersed powder is sprayed directly into the lungs. Instillation is when an aerosolized liquid is directly sprayed into the lungs. While excellent for delivering a precisely measured amount of drug to the lungs, insufflation and instillation require anesthesia of the animal. For daily drug dosing studies, this means daily anesthesia. In guinea pigs the pathway an aerosol must follow to get to the lungs is more direct and guinea pigs also have similarly sized alveoli and lung elasticity as humans [34]. This enables guinea pigs to simply inhale drugs delivered as aerosols using nose-only or whole-body chambers, without the need of anesthesia. Because the lung anatomy and physiology of guinea pigs and humans is similar, guinea pigs are the small-animal model most commonly used in preclinical studies of asthma and chronic obstructive pulmonary disease (COPD) [8]. Indeed, guinea pigs have been used to test nearly every medicine available to treat asthma [35, 36].

6.6 Guinea Pig Study Design

A typical experiment (Figure 6.1) to test an inhaled therapy will involve groups of 5–6 guinea pigs that are first infected with TB and, after time to establish infection (ex. 4 weeks), are treated daily with inhaled anti-TB drugs for an additional period (ex. an additional 4 weeks). At the end of the treatment period, the animals are sacrificed, necropsy is performed, tissue samples are sent for histopathology, and organ homogenates are plated to determine the bacterial burden in organs among the different treatment groups. The animals are also weighed regularly throughout the experiment.

Guinea pigs are docile animals that should be handled gently and quietly, as they are excitable and easily stressed. If stressed, guinea pigs will lose weight, possibly up to 50 grams in one day [37]. For this reason, it is good practice to regularly handle the guinea

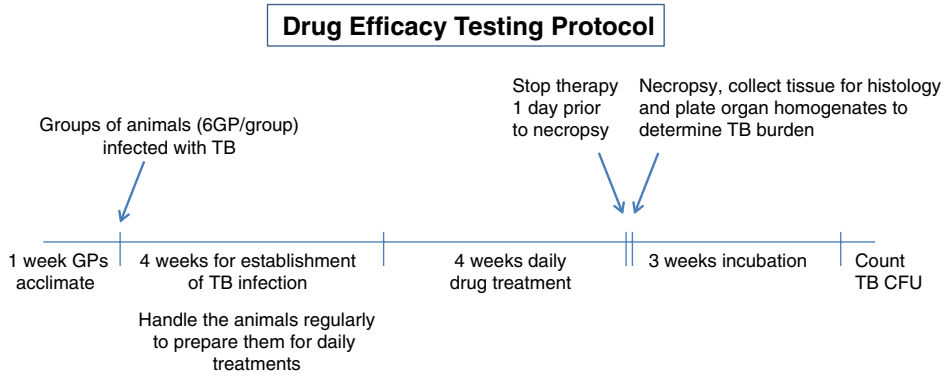


Figure 6.1 *Design of a typical experiment to test inhalation therapy for treating TB in the guinea pig (GP) model of infection*

pigs and provide food treats to them, starting from the time they arrive, to prepare them for subsequent daily dosing procedures. A list of safe treats for guinea pigs can be found at http://www.happycavy.com/food-list.htm.

6.7 Purchase and Grouping Animals

Outbred Hartley or Dunkin-Hartley guinea pigs are generally used in TB studies. These animals are commercially available from a number of suppliers (Charles River Laboratories, Hilltop Labs, and Elm Hill Labs). While the decision to use outbred guinea pigs may be driven by the lack of commercially available inbred animals, the outbred nature of these animals is advantageous for testing across a more diverse population, as is the case in the human population. Both male and female guinea pigs can be used, but mixed populations are not typically used in the same study. Hartley and Dunkin-Hartley guinea pigs are born fully furred and they gain weight rapidly after birth. A drug-efficacy study will typically last 8–12 weeks and a guinea pig with a starting weight of 300 g can reach 700–1000 g by the end of the study (Figure 6.2). In addition, because the animals are outbred, the weight of similarly aged animals can vary as much as 100 g. For this reason, it is useful to order guinea pigs by weight (225–275 g) and, after a week of acclimation, distribute comparably sized animals across the groups prior to infecting with *M. tuberculosis*. It is important to note that guinea pigs, like humans, require a dietary source of vitamin C. Commercially available guinea pig chow has sufficient vitamin C included. Mouse chow cannot be substituted, because of the lack of vitamin C.

6.8 Infecting Guinea Pigs with Virulent *Mycobacterium tuberculosis*

As the natural route of TB infection is via inhalation of a low dose of *M. tuberculosis*, it is preferable to infect animals with a low dose of bacteria using an aerosol chamber. Different chambers are available, with the most commonly used being the whole-body

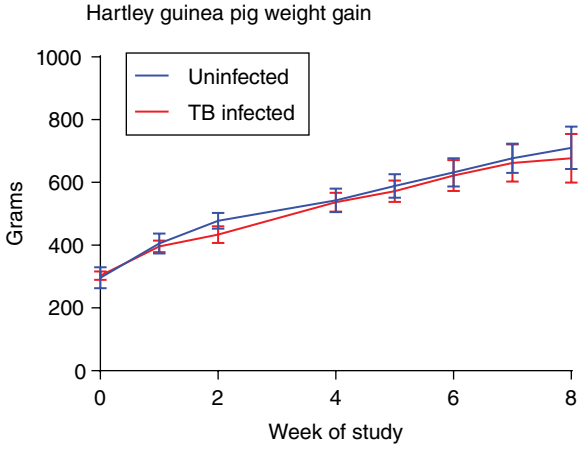


Figure 6.2 *Guinea pig weight gain over time, with or without TB infection. Male Hartley Guinea pigs were infected at week 0 with M. tuberculosis by low-dose aerosol using a Madison Chamber or were left uninfected. TB-infected group, n = 6; Uninfected group, n = 4. Average weight and standard deviations are shown*

Glas-Col aerosol chamber (Terre Haute, IN) or the Madison chamber (Madison, WI engineering workshop UW). While most groups infect with a low dose of 10–20 *M. tuberculosis* [17, 21, 38, 39], there are some drug-efficacy studies in which guinea pigs are infected with a higher aerosol dose (~200 *M. tuberculosis*) [40]. The details of infectious dose and route are important to consider when comparing studies. In addition, the virulent strain of *M. tuberculosis* that is used and its drug-sensitivity profile are other variables to consider. During the first 3 weeks of TB infection, *M. tuberculosis* grows exponentially in guinea pigs until an adaptive cell-mediated TH1 immune response is established. The hallmark of the TH1 response is the production of *M. tuberculosis* antigen-specific T cells that recognize TB and lead to the formation of granulomas that serve to control the bacterial burden [41].

6.9 Dosing Groups of Guinea Pigs with TB Drugs

In designing a drug-efficacy study, decisions need to be made about the treatment groups. All studies should include a group of animals that are untreated (i.e., *M. tuberculosis*-infected but no drug treatment). However, there are numerous possibilities for the design of the other treatment groups. Will drugs be compared following administration by different routes? Should there be a group of animals treated with standard therapy for comparison? Will new drugs be tested on their own and/or in combination with other drugs to reveal synergy? Current therapy for active TB infection is a multidrug therapy, which is important for limiting development of drug-resistant TB. Any new drug for TB would never be employed in isolation, but would be used in combination therapy [42, 43].

For standard therapy comparisons, guinea pigs can be treated with the same oral doses used in people for a combination of rifampicin, isoniazid and pyrazinamide (RHZ)

12/10/25 mg/kg [44, 45]. Daily oral dosing with this combination shows efficacy in treating *M. tuberculosis*-infected guinea pigs. However, pharmacokinetic studies of guinea pigs dosed with these concentrations of RHZ demonstrate that these drugs are poorly adsorbed from the guinea pig gut, such that the serum drug levels in guinea pigs is significantly lower than in people dosed with the same drug concentrations [43]. To achieve serum drug levels in guinea pigs that are comparable to the human levels, guinea pigs must be dosed with higher levels of RHZ; both RHZ 100/60/300 mg/kg [46] and RHZ 50/30/100 mg/kg [47] have been used. These higher doses are more effective at reducing the burden of *M. tuberculosis*. However, a side effect of oral dosing with these higher concentrations is that the natural flora in the gut is affected, creating problems for the guinea pig digestive system, such as antibiotic-associated enteritis and gastrointestinal dysbacteriosis. To offset these problems, beneficial bacteria along with extra vitamin C can be added to the diet to help reconstitute the gut microbiota and restore the animal's appetite and weight [Recipe: 20 g pumpkin puree (Libby's) + 1 g Lacto bacillus (BD Lactinex) + 12.5 g vitamin C to 80 ml 50% sucrose solution] [43, 46]. The guinea pigs like this mixture, which makes it easy to administer, and drugs can be incorporated into this mixture for hand feeding to the animals (200 µl to a 500-g guinea pig). For hand feeding guinea pigs, the animals should be held upright in their natural eating position with the opening of a 1-ml syringe placed in their mouth. Some drugs, such as rifampicin, taste bad even when mixed into this dosing cocktail, and the guinea pigs may require extra coaching to cooperate. Rifampicin has the added complication that it can interact with other drugs, including isoniazid and pyrazinamide, so it should be dosed separately and given at least 30 minutes before or after other drugs [43, 46]. Isoniazid is also sometimes given separately to avoid interactions with other drugs [48].

Delivering drugs by inhalation to guinea pigs can be done daily and is routinely performed 5 to 7 days per week. Often the treatment will take ~30 minutes a day for each animal, although some dosing chambers can accommodate more than one animal at a time (see below). For dosing, animals are placed in a chamber where the aerosol is generated. The drug is then breathed into the lungs by the normal breathing patterns of the guinea pig. It is important for the guinea pig to remain calm and relaxed in the dosing chamber. It is helpful to acclimate the animals to the chamber for 5 to 10 minutes per day for several days before actual dosing begins. Additionally, a treat can be given after dosing as positive reinforcement. Purpose-built aerosol chambers may be used in inhalation studies. When designing a chamber, it is desirable to have the guinea pig positioned in a holding tube that restricts the animal's movement but has flexible barriers that move slightly if the animal pushes on them. This helps keep the guinea pig calm so that the breathing pattern remains normal. In addition, the weight gain of animals during the course of the experiment needs to be kept in mind when designing the chamber. Openings must be small enough to contain a 300-g guinea pig but large enough to accommodate a 1 kg guinea pig. It is advantageous to design a chamber that can be adjusted for both small and large animals.

Aerosol treatment of guinea pigs may also be done by insufflation and this is the preferable method if it is necessary to know the precise amount of drug delivered to the lungs. However, insufflation requires that the animal be anesthetized. While mice can be anesthetized and insufflated daily, guinea pigs cannot tolerate it, as it is too stressful for them.

Therefore, insufflation is a method that is only useful for guinea pigs if a limited number of dosing events is required. An additional drawback of insufflation is that the anesthesia introduces an additional drug into the experiment that could possibly interact with the drugs being tested.

6.10 Collecting Data

After drug dosing is complete, the animals are sacrificed and a necropsy is performed. At the end of the dosing period it is good practice to include at least one day without drug treatment prior to necropsy to allow residual levels of drug to be metabolized by the liver and kidneys. This added day helps avoid complications arising from there being unmetabolized drug in the organ homogenates, which could affect bacterial burden measurements.

At the time of necropsy, the lungs, mediastinal lymph nodes, spleen and any other organs of interest are removed, weighed and assessed for signs of disease. It is useful to take photographs of the organs to document size and number of lesions. Weighed portions of each organ are homogenized in phosphate-buffered saline (PBS) with 0.05% Tween 80, serially diluted, and plated onto 7H10 or 7H11 Middlebrook agar with 10% Middlebrook Oleic Albumin Dextrose Catalase OADC enrichment and 0.5% glycerol (50 μg/ml carbenicillin and 0.1 mg/ml cycloheximide can be incorporated into the agar to prevent growth of non-mycobacterial contaminants). After 3 weeks incubation at 37 °C, bacterial CFU on plates are counted. Homogenization can be performed with the piece of organ placed in a Whirl-Pak bag with a mesh insert (Nasco, USA) and a Seward Stomacher (Seward Laboratory Systems Inc. USA). The mesh insert serves to hold the tissue in place as it is being homogenized. Alternatively, a Kinematica Polytron (Kinimatica Inc., Bohemia, NY) homogenizer or a Dounce homogenizer can be used. Only a portion of each organ is normally homogenized and plated and the piece/lobe taken should be consistent across animals. A piece of lung should be preserved for histology by inflating it with 10% buffered formalin using a needle syringe. Portions of the other organs may also be preserved for histology by placing them in 10% buffered formalin. For dissecting the mediastinal lymph nodes, care must be taken as these lymph nodes wrap around the trachea and, with *M. tuberculosis* infection, they can become enlarged hardened masses that are firmly attached and difficult to remove. Collected tissues can be acid-fast stained to visualize *M. tuberculosis* and hematoxylin- and eosin-stained to assess tissue histopathology and granuloma morphology.

When plating the organ homogenates it is useful to test in parallel for the presence of drug-resistant bacteria coming out of the animals. This can be determined by plating undiluted organ homogenates on 7H10/7H11 Middlebrook agar containing the drug under investigation. Drug should be incorporated into the agar at a concentration several times the minimum inhibitory concentration (MIC). If homogenates from treated animals have significantly more drug-resistant bacteria than do homogenates from untreated animals, it will indicate the ability of *M. tuberculosis* to develop drug resistance during a relatively short period of treatment.

It is standard that the data collected from an efficacy experiment include animal weight, organ histopathology, and bacterial burden in tissue. However, because of the large amount

of tissue available in these experiments there is the option to additionally perform other analysis of the drug-treated guinea pig such as metabolomics, proteomics, RNA studies, cell type recovery, or cytokine analysis.

6.11 Aerosol Dosing Chambers and Practice

Aerosol delivery for inhalation experiments in animals has a long history in the field of toxicology. Systems for reproducibly generating aerosols and exposure chambers in which these droplets or particles can be presented to animals exist and they have been reviewed previously [49, 50]. The major methods of delivery for toxicology studies are high-efficiency nebulizers for liquid-solution formulations and a variety of dry-powder dispersion systems for solid formulations. Both of these methods are designed to give extended exposures and the key parameter for consideration is the ambient concentration of aerosol from which threshold limits are derived [51].

In designing an aerosol-inhalation experiment, the first consideration is whether the drug of interest is best delivered in liquid or solid form. Collison air/gas jet nebulizers are examples of liquid-based aerosol-delivery systems used in animal studies [52]. To generate solid-based dry powders for aerosol delivery to animals, fluidized bed generators or Wrights dust feed systems [53] are traditionally used. The advantages of the above mentioned aerosol generators are that they provide: (a) known output; (b) consistency of delivery and; (c) ability to deliver for extended periods of time. These systems, however, were designed by the toxicology field and translating the knowledge for using them in safety testing to efficacy evaluation of a drug is not a simple task. The objectives of drug-efficacy testing differ from those of toxicology in important respects. In particular, efficacy testing is focused on bolus dose response. Consequently, delivery and exposure systems for drug-efficacy studies ideally consider: (a) dose delivered (accuracy); (b) duration of delivery (preferably as short as possible); (c) consistency of delivery (reproducibility).

A number of additional factors must also be considered when developing a method of aerosol delivery for testing an inhaled drug therapy.

- Study timing with regard to scale of manufacturing
- Animal model selection
- Dose and dosing regimen

6.11.1 Study Timing with Regard to Scale of Manufacturing

The timing of a study needs to be considered in the framework of the larger scope of development activities (early development or late development). If there is not sufficient drug supply to support a classical toxicological approach to delivery and exposure (i.e., prior pharmacokinetic studies), should early testing by inhalation be performed? If drug supply is limiting, it may necessitate the use of a smaller dosing chamber with higher delivery efficiency to the animal. Additionally, a small drug supply may limit the number of animals tested and different routes of delivery that can be compared. Later in development, after the drug supply has moved from production in a research facility to large-scale production in a manufacturing facility, the supply is not as limited and there are more options for experimental design.

6.11.2 Animal Model Selection

A variety of animal models are available for both safety and efficacy testing with aerosols. Often these studies begin with the rat model. However, in TB research the mouse and guinea pig models are the most popular small-animal models (see Chapter 3). As described above, there are significant differences between mice and guinea pigs in terms of pulmonary function [54] and anatomy [55]. Owing to the size of the airways and small tidal volume the mouse is not the best model for aerosol delivery, although screening by instillation or insufflation may be a valuable approach. On the other hand, guinea pigs have sufficient potential for lung deposition and are a well-accepted model for *M. tuberculosis* infection, which renders them the preferred animal model for testing inhaled-drug therapies to treat TB.

6.11.3 Dose and Dosing Regimen

The dose of drug delivered to the animal is limited by tolerability and should also be selected according to the dose that can realistically be delivered clinically. The upper limit of aerosol dosing is currently considered to be approximately 100 mg/person [56]. As a working range, based on a standard 70-kg person, a 1–2 mg/kg dose would translate into approximately 70–140 mg/person. It is desirable, therefore, to generate aerosols in airborne concentrations sufficient to deposit this dose in the animal model.

Dosing can be approximated from exposure calculations [57, 58]. The dose calculation is based primarily on particle size, concentration of aerosol in air, tidal volume and breathing frequency, but particle distribution (proportion in various sizes) and concentration of drug in droplets or particles are additional considerations. If resources allow, it is desirable to confirm the dosing estimate through pharmacokinetic studies of animals that inhale the aerosol dose compared with animals receiving known doses directly delivered to the lungs by intratracheal spray instillation or insufflation [57–59]. This allows the aerosol dosing protocol to be confirmed by direct comparison to tissue drug levels after delivery of a known dose directly to the lungs.

6.12 Nebulizer Aerosol Delivery Systems for Liquids

In recent years, high-concentration low-gas-volume vibrating mesh nebulizer systems have become available that facilitate delivery of accurate doses without wasting drug to exhaust as occurs with jet-nebulized aerosols [60]. Figure 6.3a depicts the operation of the piezoelectric mesh (Aerogen, Galway, Ireland) delivering droplets and a section through a single orifice showing the liquid delivery. Droplets in respirable sizes of approximately 1–5 μm are produced by this system. Figure 6.3b illustrates the concentration of aerosol droplets in air as delivered to a fixed volume such as an exposure chamber from which animals inhale to receive the dose of delivered drug. With this system, a steady state is achieved resulting in controlled dosing for the duration of aerosol delivery.

Classical nose-only exposure chambers, as illustrated in Figure 6.4, are ideal for delivery of nebulized drugs to guinea pigs. However, the type of nebulizer and size of the chamber will influence the amount of drug required for the study. For air jet nebulizers, such as the Collison, the airflow required to run through the chamber is frequently in the range 8–12 l/min, while vibrating mesh systems can require airflow as low as 2 l/min for aerosol delivery. As a result the vibrating mesh systems use much less drug to deliver the target dose.

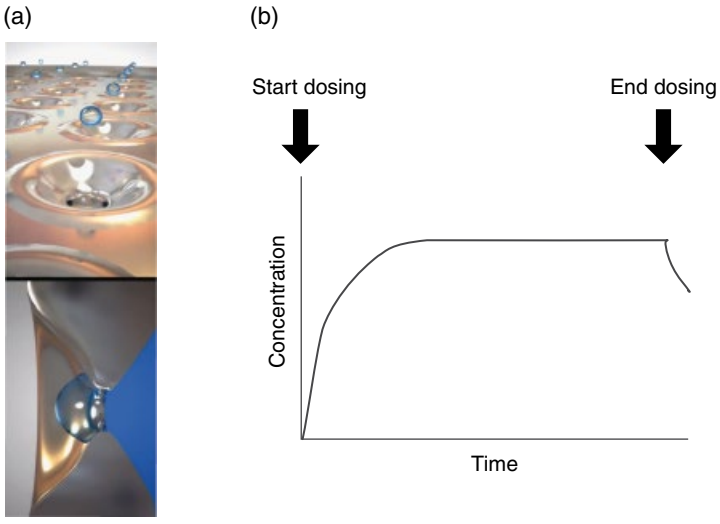


Figure 6.3 *(a) Vibrating mesh nebulizer. A mesh of laser-drilled holes vibrating at sonic frequencies acts as micro-pumps to generate droplets. Image courtesy of Aerogen (Galway, Ireland). (b) Plot of ambient concentration of aerosol (drug) in confined volume (exposure chamber) as a function of time of dosing*

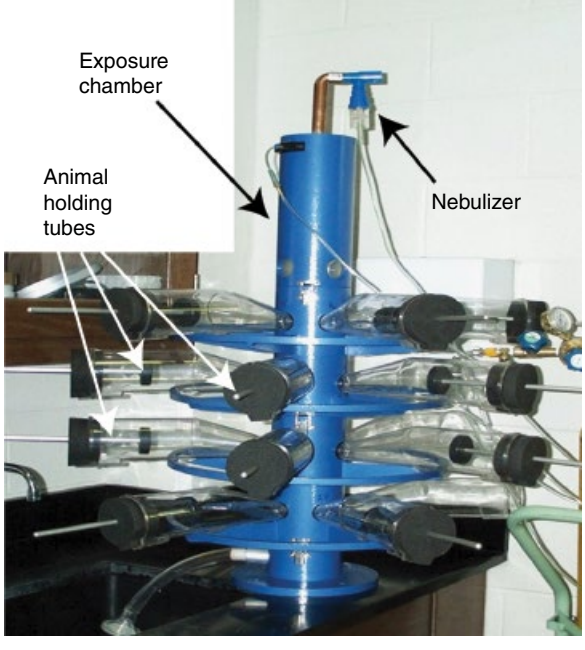


Figure 6.4 *Multi-port nose-only exposure chamber in which animals are arranged around a central exposure chamber and breathe directly from the reservoir of aerosol*

6.13 Dry-Powder Aerosol Delivery Systems for Solids

Powder formulation is an important factor in aerosol delivery in the solid state. Most frequently, drug is micronized into a size range of 1–5 μm, suitable for delivery [60]. This process requires large amounts of drug and many times yields particles that do not have good dispersion properties. Recently, spray-dried particles have been developed that have excellent dispersion properties and require low energy input for efficient generation [21], which allows dispersion of very small doses. These spray dryers have unique technology to control the airflow on which the aerosol is carried. There are several systems available commercially; Buchi (Switzerland) makes small bench top spray dryers, capable of making small quantities of spray-dried particles with little wasted drug, and Niro (Columbia, MD) and Vertis (UK) make larger spray dryers.

Two approaches are available for delivering spray-dried particles. The first approach employs recirculation of the doses, assuming drug supply is not overly constrained [11]. This approach uses a fan at the base of the exposure chamber to maintain a static cloud of dry powder (Figure 6.5) and results in steady-state dispersion similar to that illustrated in Figure 6.3b and, consequently, will not be discussed further. The second method is ideal for dosing when drug is in short supply. Owing to the excellent dispersion properties of spray-dried powders a simple method of aerosol generation can be adopted as illustrated in Figure 6.6a, consisting of a powder reservoir through which air can be driven from a syringe pushing the powder under high-shear conditions through a needle into the desired exposure chamber (confined volume) (P. Durham and A.J. Hickey, unpublished data). This approach requires manual delivery of the powder through the needle at regular intervals (ex. 2-minute intervals) during the dosing period. Figure 6.6b illustrates the concentration of drug in the chamber resulting from this method of dosing. In a sequential fashion, drug is delivered and subsequently depleted due to removal in the breath of the animal as well as sedimentation. This method of dosing provides an option when drug supply is limited and steady-state exposure is not feasible.

When dosing a drug that is in short supply, the exposure chamber in which the animals are placed for dry-powder delivery requires two important features (Figure 6.7). First, the exposure chamber should have a small volume, which can be saturated with small quantities of drug. Second, orifices through which air can enter the exposure chamber from the

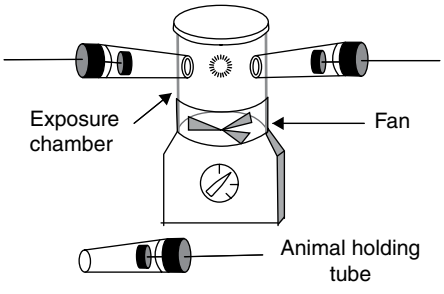


Figure 6.5 *A dry-powder aerosol delivery system with three nose ports for securing animal-holding tubes during exposure. A fan at the base of the chamber generates a static cloud of dispersed powder within the exposure chamber*

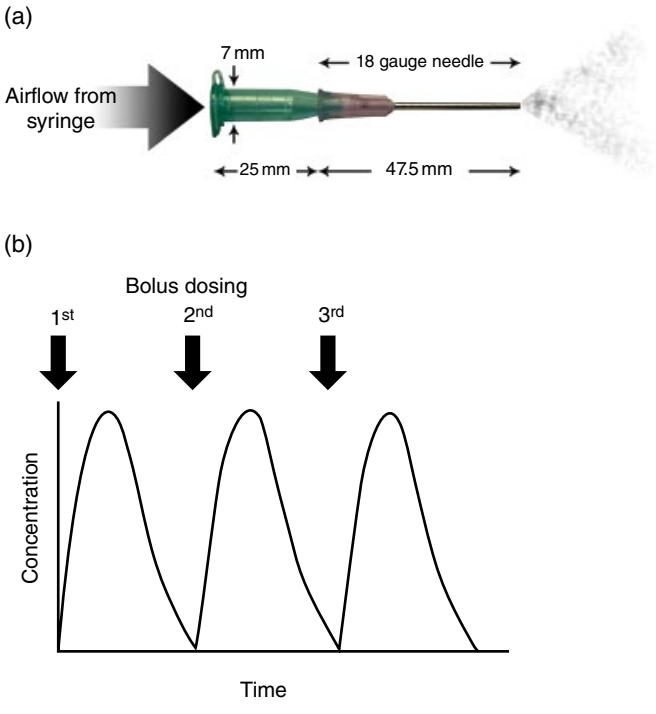


Figure 6.6 *(a) A dry-powder dosator for aerosol dispersion and (b) plot of ambient concentration of aerosol (drug) in confined volume (exposure chamber) as a function of time of dosing*

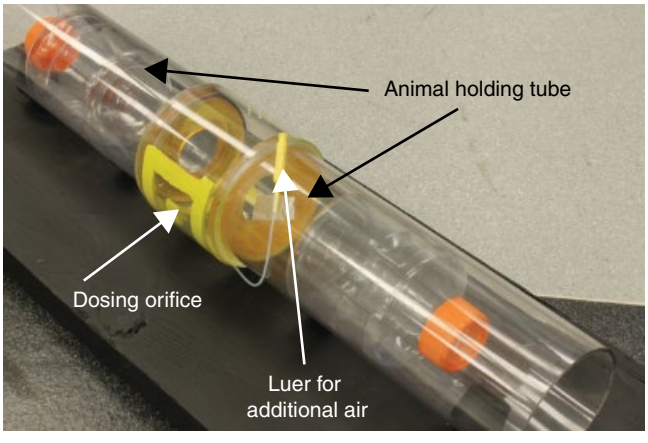


Figure 6.7 *A purpose-built chamber for two animals with a central exposure chamber into which dry-powder aerosols can be introduced through a syringe at the dosing orifice. The orange cap bottles in this device serve as the flexible barrier that contains the animal during dosing. Luer and narrow tubing shown allow active introduction of small volumes of air from a syringe. There are also small orifices in the middle chamber that allow for gas exchange and passive air intake in response to breathing*

atmosphere, in response to animal breathing frequency and tidal volume, should be present. Additional features include the aerosol dosing port through which drug can be introduced and a means of introducing small volumes of air with a syringe should the animals require additional air supply during dosing. These features serve to maximize the drug delivered to the animal while minimizing the amount of drug needed for the experiment.

6.14 Summary

An inhaled TB drug holds promise for expanding and improving the current treatment options for this dreaded disease. The guinea pig is the preferred animal model for evaluating such therapies. To test the efficacy of an inhaled drug for TB requires expertise in *M. tuberculosis* pathogenesis, animal models of disease, drug formulation and pulmonary delivery. In the future, we hope that this combination of knowledge and skills will become more accessible and enable routine testing of all new TB drugs as inhaled therapies.

Acknowledgments

We wish to thank past and present members of the Braunstein and Hickey laboratories for help on projects to test inhaled drug therapies for TB, most recently Lucila Garcia-Contreras, Phil Durham, Jenny Hayden, Seidu Malik, Brittany Miller, Ellen Perkowski, and Kate Zulauf. Support for this work in the Braunstein and Hickey laboratories comes from the National Institutes of Health NIAID and the AIDS Clinical Trials Group.

References

[1] CDC (2003) *CDC Morbidity and Mortality Weekly Report*, pp. 1–77.
[2] WHO (2015) *Tuberculosis fact sheet* http://www.who.int/mediacentre/factsheets/fs104/en/(accessed 13th March 2016).
[3] Migliori, G.B., Lange, C., Centis, R. *et al*. (2008) Resistance to second-line injectables and treatment outcomes in multidrug-resistant and extensively drug-resistant tuberculosis cases. *Eur. Respir. J.*, **31**(6), 1155–1159.
[4] Furin, J., Isaakidis, P., Reid, A.J. *et al*. (2014) 'I'm fed up': experiences of prior anti-tuberculosis treatment in patients with drug-resistant tuberculosis and HIV. *Int. J. Tuberc. Lung Dis.*, **18**(12), 1479–1484.
[5] Muttil, P., Wang, C. and Hickey, A.J. (2009) Inhaled drug delivery for tuberculosis therapy. *Pharm. Res.*, **26**(11), 2401–2416.
[6] Misra, A., Hickey, A.J., Rossi, C. *et al*. (2011) Inhaled drug therapy for treatment of tuberculosis. *Tuberculosis (Edinburgh)*, **91**(1), 71–81.
[7] Mortensen, N.P., Durham, P. and Hickey, A.J. (2014) The role of particle physico-chemical properties in pulmonary drug delivery for tuberculosis therapy. *J. Microencapsul.*, **31**(8), 785–795.
[8] Canning, B.J. (2003) Modeling asthma and COPD in animals: a pointless exercise? *Curr. Opin. Pharmacol.*, **3**(3), 244–250.
[9] Assael, B.M., Pressler, T., Bilton, D. *et al*. (2013) Inhaled aztreonam lysine vs. inhaled tobramycin in cystic fibrosis: A comparative efficacy trial. *J. Cyst. Fibros.*, **12**(2), 130–140.
[10] Littlewood, K.J., Higashi, K., Jansen, J.P. *et al*. (2012) A network meta-analysis of the efficacy of inhaled antibiotics for chronic Pseudomonas infections in cystic fibrosis. *J. Cyst. Fibros.*, **11**(5), 419–426.

[11] Hickey, A.J. (2004) Summary of common approaches to pharmaceutical aerosol administration, in *Pharmaceutical Inhalation Aerosol Technology* (ed. A.J. Hickey), Marcel Dekker, Inc., New York, NY, pp. 385–421.
[12] Monforte, V., Ussetti, P., López, R. *et al*. (2009) Nebulized Liposomal Amphotericin B Prophylaxis for Aspergillus Infection in Lung Transplantation: Pharmacokinetics and Safety. *J. Heart Lung Transplant.*, **28**(2), 170–175.
[13] Shirk, M.B., Donahue, K.R. and Shirvani, J. (2006) Unlabeled uses of nebulized medications. *Am. J. Health Syst. Pharm.*, **63**(18), 1704–1716.
[14] Miller, J.B., Abramson, H.A. and Ratner, B. (1950) Aerosol streptomycin treatment of advanced pulmonary tuberculosis in children. *Am. J. Dis. Child.*, **80**(2), 207–237.
[15] Dartois, V. (2014) The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. *Nat. Rev. Microbiol.*, **12**(3), 159–167.
[16] Suarez, S., O'Hara, P., Kazantseva, M. *et al*. (2001) Airways delivery of rifampicin microparticles for the treatment of tuberculosis. *J. Antimicrob. Chemother.*, **48**(3), 431–434.
[17] Garcia-Contreras, L., Sethuraman, V., Kazantseva, M. *et al*. (2006) Evaluation of dosing regimen of respirable rifampicin biodegradable microspheres in the treatment of tuberculosis in the guinea pig. *J. Antimicrob. Chemother.*, **58**(5), 980–986.
[18] Pandey, R., Sharma, A., Zahoor, A. *et al*. (2003) Poly (DL-lactide-co-glycolide) nanoparticle-based inhalable sustained drug delivery system for experimental tuberculosis. *J. Antimicrob. Chemother.*, **52**(6), 981–986.
[19] Sharma, A., Sharma, S. and Khuller, G.K. (2004) Lectin-functionalized poly (lactide-co-glycolide) nanoparticles as oral/aerosolized antitubercular drug carriers for treatment of tuberculosis. *J. Antimicrob. Chemother.*, **54**(4), 761–766.
[20] Pandey, R. and Khuller, G.K. (2005) Solid lipid particle-based inhalable sustained drug delivery system against experimental tuberculosis. *Tuberculosis (Edinburgh)*, **85**(4), 227-234.
[21] Garcia-Contreras, L., Fiegel, J., Telko, M.J. *et al*. (2007) Inhaled large porous particles of capreomycin for treatment of tuberculosis in a guinea pig model. *Antimicrob. Agents Chemother.*, **51**(8), 2830–2836.
[22] Lee, R.E., Hurdle, J.G., Liu, J. *et al*. (2014) Spectinamides: a new class of semisynthetic antituberculosis agents that overcome native drug efflux. *Nat. Med.*, **20**(2), 152–158.
[23] Roy, C.J., Sivasubramani, S.K., Dutta, N.K. *et al*. (2012) Aerosolized gentamicin reduces the burden of tuberculosis in a murine model. *Antimicrob. Agents Chemother.*, **56**(2), 883–886.
[24] Sacks, L.V., Pendle, S., Orlovic, D. *et al*. (2001) Adjunctive salvage therapy with inhaled aminoglycosides for patients with persistent smear-positive pulmonary tuberculosis. *Clin. Infect. Dis.*, **32**(1), 44–49.
[25] Hokey, D.A., Wachholder, R., Darrah, P.A. *et al*. (2014) A nonhuman primate toxicology and immunogenicity study evaluating aerosol delivery of AERAS-402/Ad35 vaccine: Evidence for transient t cell responses in peripheral blood and robust sustained responses in the lungs. *Hum. Vaccine Immunother.*, **10**(8), 2199–2210.
[26] Muttil, P., Kaur, J., Kumar, K. *et al*. (2007) Inhalable microparticles containing large payload of anti-tuberculosis drugs. *Eur. J. Pharm. Sci.*, **32**(2), 140–150.
[27] Padilla-Carlin, D.J., McMurray, D.N. and Hickey, A.J. (2008) The guinea pig as a model of infectious diseases. *Comp. Med.*, **58**(4), 324–340.
[28] Saini, D., Hopkins, G.W., Seay, S.A. *et al*. (2012) Ultra-low dose of Mycobacterium tuberculosis aerosol creates partial infection in mice. *Tuberculosis (Edinburgh)*, **92**(2), 160–165.
[29] McMurray, D.N. (2001) Disease model: pulmonary tuberculosis. *Trends Mol. Med.*, **7**(3), 135–137.
[30] Orme, I.M. and Basaraba, R.J. (2014) The formation of the granuloma in tuberculosis infection. *Semin. Immunol.*, **26**(6), 601–609.
[31] Harper, J., Skerry, C., Davis, S.L. *et al.* (2012) Mouse model of necrotic tuberculosis granulomas develops hypoxic lesions. *J. Infect. Dis.*, **205**(4), 595–602.
[32] Driver, E.R., Ryan, G.J., Hoff, D.R. *et al*. (2012) Evaluation of a Mouse Model of Necrotic Granuloma Formation Using C3HeB/FeJ Mice for Testing of Drugs against Mycobacterium tuberculosis. *Antimicrob. Agents Chemother.*, **56**(6), 3181–3195.

[33] Mitchison, D.A. and Davies, G.R. (2008) Assessment of the Efficacy of New Anti-Tuberculosis Drugs. *Open Infect. Dis. J.*, **2**, 59–76.
[34] Amdur, M.O. and Mead, J. (1958) Mechanics of respiration in unanesthetized guinea pigs. *Am. J. Physiol.*, **192**(2), 364–368.
[35] Canning, B.J. and Chou, Y. (2008) Using guinea pigs in studies relevant to asthma and COPD. *Pulm. Pharmacol. Ther.*, **21**(5), 702–720.
[36] Evans, R.L., Nials, A.T., Knowles, R.G. *et al*. (2012) A comparison of antiasthma drugs between acute and chronic ovalbumin-challenged guinea-pig models of asthma. *Pulm. Pharmacol. Ther.*, **25**(6), 453–464.
[37] Banks, R. (1989) The Guinea Pig: Biology, Care, Identification, Nomenclature, Breeding and Genetics, in *USAMRIID Seminar Series*.
[38] Aiyaz, M., Bipin, C., Pantulwar, V. *et al*. (2014) Whole genome response in guinea pigs infected with the high virulence strain Mycobacterium tuberculosis TT372. *Tuberculosis (Edinburgh)*, **94**(6), 606–615.
[39] Smith, D.W., McMurray, D.N., Wiegeshaus, E.H. *et al*. (1970) Host-parasite relationships in experimental airborne tuberculosis. IV. Early events in the course of infection in vaccinated and nonvaccinated guinea pigs. *Am. Rev. Respir. Dis.*, **102**(6), 937–949.
[40] Dutta, N.K., Illei, P.B., Peloquin, C.A. *et al*. (2012) Rifapentine is not more active than rifampin against chronic tuberculosis in guinea pigs. *Antimicrob. Agents Chemother.*, **56**(7), 3726–3731.
[41] Orme, I.M. and McMurray, D.N. (1996) The immune response to tuberculosis in animal models, in *Tubercuosis* (eds W.N. Rom and S. Garay), Little, Brown, Boston.
[42] Dhillon, J., Dickinson, J.M., Sole, K. *et al*. (1996) Preventive chemotherapy of tuberculosis in Cornell model mice with combinations of rifampin, isoniazid, and pyrazinamide. *Antimicrob. Agents Chemother.*, **40**(3), 552–555.
[43] Ordway, D.J., Shanley, C.A., Caraway, M.L. *et al*. (2010) Evaluation of standard chemotherapy in the guinea pig model of tuberculosis. *Antimicrob. Agents Chemother.*, **54**(5), 1820–1833.
[44] Johnson, C.M., Pandey, R., Sharma, S. *et al*. (2005) Oral therapy using nanoparticle-encapsulated antituberculosis drugs in guinea pigs infected with Mycobacterium tuberculosis. *Antimicrob. Agents Chemother.*, **49**(10), 4335–4338.
[45] Lenaerts, A.J., Hoff, D., Aly, S. *et al*. (2007) Location of persisting mycobacteria in a Guinea pig model of tuberculosis revealed by r207910. *Antimicrob. Agents Chemother.*, **51**(9), 3338–3345.
[46] Dutta, N.K., Alsultan, A., Gniadek, T.J. *et al*. (2013) Potent rifamycin-sparing regimen cures guinea pig tuberculosis as rapidly as the standard regimen. *Antimicrob. Agents Chemother.*, **57**(8), 3910–3916.
[47] Shang, S., Shanley, C.A., Caraway, M.L. *et al*. (2012) Drug treatment combined with BCG vaccination reduces disease reactivation in guinea pigs infected with Mycobacterium tuberculosis. *Vaccine*, **30**(9), 1572–1582.
[48] Grosset, J., Truffot-Pernot, C., LaCroix, C. *et al*. (1992) Antagonism between isoniazid and the combination pyrazinamide-rifampin against tuberculosis infection in mice. *Antimicrob. Agents Chemother.*, **36**(3), 548–551.
[49] Leong, B.K.J. (1981) *Inhalation Toxicology and Technology*. Ann Arbor Science, Ann Arbor, MI.
[50] Jaeger, R.J., Shami, S.G. and Tsenova, L. (2006) Directed-flow aerosol inhalation exposure systems: Applications to pathogens and highly toxic agents, in *Inhalation Toxicology* (ed. H.S.a.S.A. Katz), CRC Taylor Francis Group, New York, pp. 73–90.
[51] Hinds, W.C. (1999) Threshold limit values, in *Aerosol Technology, Properties, Behavior and Measurement of Airborne Particles*, John Wiley and Sons, NY, p. 226.
[52] May, K.R. (1973) The Collison nebulizer: description, performance and application. *J. Aerosol Sci.*, **4**, 235–238.
[53] Hinds, W.C. (1999) Production of test aerosols, in *Aerosol Technology, Properties, Behavior and Measurement of Airborne Particles*, John Wiley and Sons, NY, pp. 428–446.
[54] Chaffee, V.W. (1974) Surgery of laboratory animals, in *Handbook of Laboratory Animal Science*, (eds E.C. Melby and N.H. Altman), CRC Press, Cleveland, OH.

[55] Tran, L. and Kuempel, E. (2007) Biological based lung dosimetry and exposure-dose-response models for poorly soluble inhaled particles, in *Particle Toxicology* (ed. K.D.a.P. Borm), CRC Press Taylor and Francis Group, Boca Raton, Fl, pp. 351–386.

[56] Geller, D.E., Weers, J. and Heuerding, S. (2011) Development of an inhaled dry-powder formulation of tobramycin using PulmoSphere technology. *J. Aerosol. Med. Pulm. Drug Deliv.*, **24**(4), 175–182.

[57] Cryan, S.A., Sivadas, N. and Garcia-Contreras, L. (2007) In vivo animal models for drug delivery across the lung mucosal barrier. *Adv. Drug Deliv. Rev.*, **59**(11), 1133–1151.

[58] Suarez, S., Kazantseva, M., Bhat, M. *et al*. (2001) The influence of suspension nebulization or instillation on particle uptake by guinea pig alveolar macrophages. *Inhal. Toxicol.*, **13**(9), 773–788.

[59] Suarez, S. and Hickey, A.J. (2001) Pharmacokinetic and pharmacodynamic aspects of inhaled therapeutic agents, in *Medical Applications of Computer Modelling: The Respiratory System* (ed. T. Martonen), WIT Press, Southampton, UK, pp. 225–304.

[60] Dhand, R. (2002) Nebulizers that use a vibrating mesh or plate with multiple apertures to generate aerosol. *Respir. Care*, **47**(12), 1406-1416; discussion 1416–1418.

7

Preclinical Pharmacokinetics of Antitubercular Drugs

Mariam Ibrahim and Lucila Garcia-Contreras

College of Pharmacy, the University of Oklahoma Health Sciences Center, Oklahoma City, OK

7.1 Introduction

Tuberculosis (TB) infection is still considered to be a threat to public health in the world, despite the major progress achieved in most world regions by implementation of the directly observed treatment short-course (DOTS). In industrialized nations, the main TB-related health crisis is due to the high morbidity and mortality rates among HIV patients in which TB causes one out of four deaths [1]. In sub-Saharan Africa and in parts of Eastern Europe where the DOTS system has not been implemented, the main challenge to the control of TB infection is patient compliance and improper dose/dosing frequency prescription [1]. Together, these factors result in drug concentrations lower than the minimum inhibitory concentration (MIC) at the target site, which in turn yields to the emergence of drug-resistant strains, multi-drug-resistant and extensively-drug-resistant TB. Surprisingly, even in regions of the world in which the DOTS strategy has been implemented successfully in adults, TB control is still a challenge in children. Hiruy *et al*. reported that only a fraction of the children receiving the standard DOTS treatment in a South African hospital reached therapeutic concentrations of first-line agents, as defined by the World Health Organization (WHO) [2]. This study highlights the importance of monitoring drug levels to have a better control of the infection.

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

In general, a full understanding of the pharmacokinetic (PK) behavior of each anti-TB drug and drug combinations is essential to determine the optimum dose/dosing regimen for a specific patient population. This information is also required to establish pharmacokinetic–pharmacodynamic (PK–PD) profile to evaluate their efficacy in TB treatment and to reduce the emergence of drug-resistant strains. In order to fully characterize the PK behavior of a drug, it is necessary to know its physicochemical properties, the best formulation and route of administration to enhance its therapeutic potential and to select the most suitable animal model. The biopharmaceutical and PK properties of each drug will determine the drug-distribution and -elimination mechanisms after administration and its concentration at the target tissue. These factors will determine the success of therapy in decreasing bacterial burden. This chapter focuses on the preclinical pharmacokinetics of anti-TB drugs, discussing the elements that in our opinion should be included in preclinical PK studies, from selection of a suitable animal model to the computational PK analysis and its correlation with relevant pharmacodynamics (PD) parameters that ultimately determine efficacy of treatment. Comparative case studies of selected anti-TB drugs illustrating all the discussed factors are also provided.

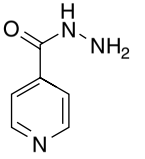
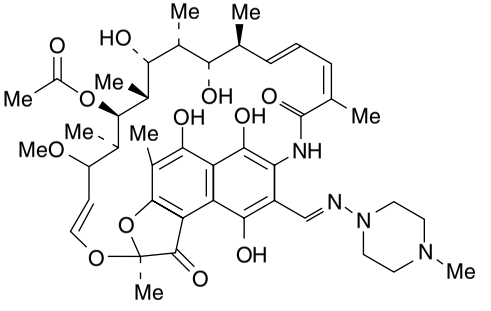
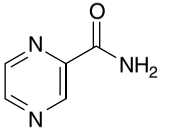
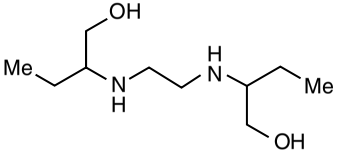
7.2 Factors Influencing the Pharmacokinetic Behavior of Drugs

7.2.1 Physicochemical Properties of the Drug

The main physicochemical factors that affect the absorption, distribution and elimination of a drug include molecular weight, aqueous solubility and Log *P*, which is defined as the partition coefficient ratio of concentrations of un-ionized drug between two liquid phases. Generally a higher Log *P* value corresponds to a hydrophobic drug and a small Log P corresponds to a hydrophilic drug. Drugs having a high Log *P* value can be more easily absorbed through membranes due to lipophilicity but their elimination rate will be slower. The numeric values of these parameters for first- and second-line anti-TB agents are listed in Table 7.1. Based on these parameters and drug permeability, these drugs have been further grouped in the different classes of the biopharmaceutical classification system (BCS) that the WHO has used to grant bio-wavers to establish the interchangeability of suppliers [16].

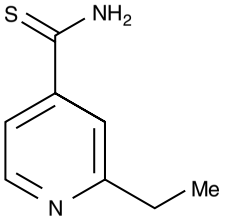
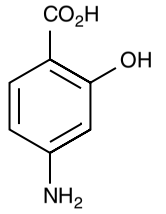
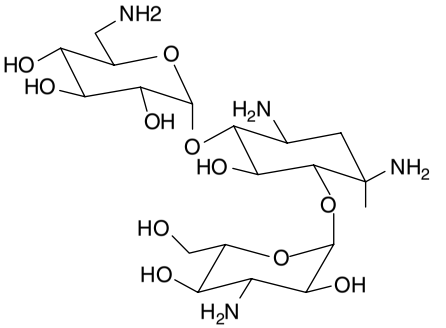
The first-line agents, isoniazid and pyrazinamide, are among the compounds with the smallest molecular weight, are soluble in water, and have Log *P* values between −1.884 and −0.7. Depending on the limit of permeability used (85 or 90%), they may be considered to be in the class I (high solubility and high permeability) or class III (high solubility, low permeability) of the BCS system and they have an oral bioavailability greater than 90% (Table 7.2). In contrast, rifampicin has a larger molecular weight, very limited water solubility and a Log $P = 4.0$. Thus, rifampicin is considered to belong to the BCS class II (low solubility and high permeability) and has an oral bioavailability greater than 90% (Table 7.2). However, its pH-dependent solubility is known to affect its absorption from the gastrointestinal (GI) tract. In contrast, most second-line agents have higher molecular weights and are water soluble. The oral bioavailability of these agents is low, so they are given by injection in order to improve their absorption and most of them are not considered by the BCS classification.

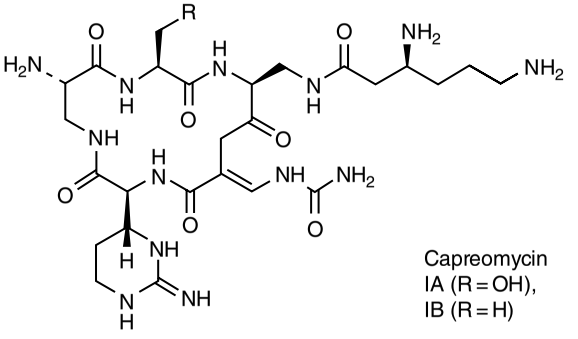
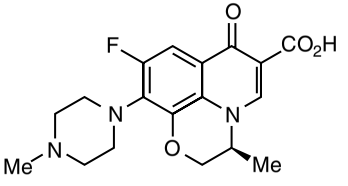
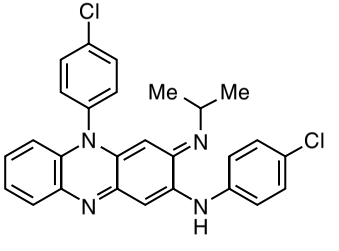
Table 7.1 *Chemical structure and selected physicochemical properties of first- and second-line anti-TB drugs*

Drug	Molecular structure	Mol. wt. (g/mol)	Aqueous solubility (mg/ml)	Polarity Log *P*	Ref.
Isoniazid		137.14	Freely soluble (140)	–0.7	[3]
Rifampicin		822.94	Slightly soluble (1–2)	4.0	[4]
Pyrazinamide		123.11	Sparingly soluble (15)	–1.884	[5]
Ethambutol		204.31	Sparingly soluble (10)	–0.14	[6, 7]

(*continued*)

Table 7.1 *(Continued)*

Drug	Molecular structure	Mol. wt. (g/mol)	Aqueous solubility (mg/ml)	Polarity Log *P*	Ref.
Ethionamide		166.24	Very slightly soluble (0.1)	0.705	[8]
p-Aminosalicylic acid		153.14	Slightly soluble (1.7)	1.012	[9]
Kanamycin A		582.58	Soluble (50)	−7.936	[10]

Capreomycin		766.786	Soluble (50)	−9.609	[11]
Levofloxacin		361.37	Soluble (100)	1.268	[12]
Clofazimine		473.40	Insoluble (0.01)	7.132	[13]

(*continued*)

Table 7.1 *(Continued)*

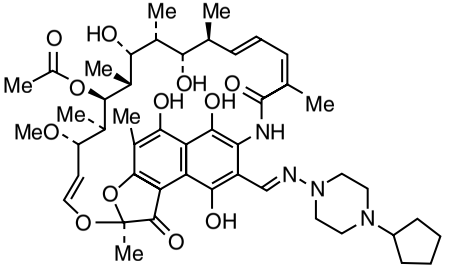
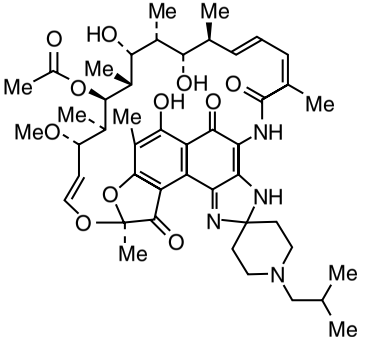
Drug	Molecular structure	Mol. wt. (g/mol)	Aqueous solubility (mg/ml)	Polarity Log *P*	Ref.
Rifapentine		877.031	Very slightly soluble (<1)	5.29	[14]
Rifabutin		847.005	Very slightly soluble (0.19)	4.218	[15]

Table 7.2 *Clinical and pharmacokinetic parameters for the first- and second-line anti-TB drugs in humans*

Drug	Route of administration	Protein binding (%)	Bioavailability (%)	Half-life (h)
Isoniazid	oral, IM or IV	0–10 [17]	Close to 100 [3]	0.5–1.6
Rifampicin	oral or IV	80–89 [18]	90–95 [19]	3–4
Pyrazinamide	oral	10–20 [20] [5]	>90	9.6 ± 1.8 [5]
Ethambutol	oral	20–30 [21]	~80 [22]	3–4
Streptomycin	IM or IV	30–34 [23, 24]	84–88 [25]	5–6
Ethionamide	Oral, IV or IM	~30 [26]	Close to 100	1.92 [8]
Capreomycin	IM or IV	~30 [27]	Not orally bioavailable [11]	0.18 ± 0.05 [28]
Moxifloxacin	oral or IV	39.4 ± 2.4	90 [29]	11.5–15.6
Levofloxacin	oral or IV	24–38	~100	7.7–8.9
Clofazimine	oral	—	45–62 [30]	70 days [13]
Cycloserine	oral	<20	~70 to 90	10

7.2.2 Formulation and Routes of Administration

Anti-TB drugs are mainly administered by oral and parenteral routes. Table 7.2 lists the route of administration for representative anti-TB drugs, their doses and some of the clinical and pharmacokinetic parameters in humans. Anti-TB drugs are currently available as tablets, capsules and granules and as lyophilized powder for reconstitution or solution for injection. Drugs given by the oral route as tablets (isoniazid, pyrazinamide, ethambutol, ethionamide) would require more time for disintegration and dissolution compared with capsules (rifampicin, cycloserine). *p*-Aminosalicylic acid (PAS) is available as enteric coated granules to prevent the release of drug in stomach without effecting any serum drug concentration [31]. These formulation differences and the varying times to be dissolved may have implications for their absorption and residence time in the GI tract, which ultimately may affect their bioavailability.

Drugs that have poor oral bioavailability are administered by the parenteral route as injected drug solutions since they would be expected to have higher bioavailability as there would be fewer barriers to their absorption. Parenteral routes are also employed when a faster onset of action is desired. Drugs in solution for intravenous (IV) injection such as streptomycin and capreomycin) would have higher bioavailability (almost 100%) compared with injections by intramuscular (IM) route (Table 7.2). In the last decade,

pulmonary delivery has proven to be an attractive route of administration for treatment of TB because drugs administered by this route achieve high local concentration in the lungs, can be used to target macrophages, and result in reduced systemic toxicity.

7.2.3 Disease State

TB treatment is further complicated when the patient experiences simultaneous diseases such as HIV, diabetes, or liver and/or kidney dysfunction. Concurrent treatments for these co-morbid diseases may cause failure or relapse of TB treatment. HIV-positive patients are more prone to develop TB infection as well as other parallel opportunistic infections [32]. Thus, these patients may need to take multiple drugs at the same time, which may cause incomplete absorption of one or more drugs in the regimen [33]. There is also a significant risk of drug–drug interactions, such as those observed between anti-HIV and anti-TB drugs. Notably, rifamycins are potent inducers of cytochrome P450, an important hepatic metabolizing enzyme.

When diabetic patients are exposed to TB, they are more likely to progress to active disease, and diabetes would hamper the efficacy of TB treatment in these patients. For example, diabetic patients often present with gastroparesis, a GI problem that can lead to either delayed drug absorption or incomplete absorption. Diabetic patients may also present other medical conditions including coronary artery disease or chronic renal failure that delay both metabolism and elimination anti-TB drugs.

Compared with patients with normal renal function, those with renal failure undergoing dialysis are more likely to have active TB either as a consequence of a latent infection or a new exposure. Patients with renal failure are at risk of experiencing drug accumulation leading to adverse drug reactions. Specifically, drugs that are excreted mainly in the urine (ethambutol, cycloserine, aminoglycosides) pose a bigger risk as they would be accumulated to a larger extent. The same cautionary approach needs to be taken for pyrazinamide, whose metabolites, pyrazinoic acid and 5-hydroxypyrazinoic acid, are also excreted in urine. Likewise, hepatic dysfunction decreases clearance of drugs such isoniazid, rifampicin, pyrazinamide, ethionamide, and PAS. Moreover, patients with hepatic or renal dysfunctions often suffer from both nausea and vomiting. Thus, they may experience both limited absorption and reduced drug clearance.

7.3 Pulmonary Delivery of Anti-TB Drugs

Several research groups have studied the possibility of delivering drugs directly to the lungs for TB treatment [34–39]. Pulmonary delivery brings the drug in close vicinity with the bacteria and increases local concentrations of the drug in the lungs. This therapeutic approach may allow the use of smaller drug doses to achieve a therapeutic effect in the lungs and reduce the systemic side effects associated with their use. In addition, pulmonary delivery would be advantageous for drugs that are subject to first-pass metabolism, due to the limited enzymatic activity in the lungs compared with the liver [40]. In addition, the alveolar region offers an extensive area for drug absorption, thin membrane and abundant vasculature, which is likely to increase the fraction of drug absorbed. Furthermore, the presence of lung surfactant in the epithelial lining fluid can facilitate the dissolution of

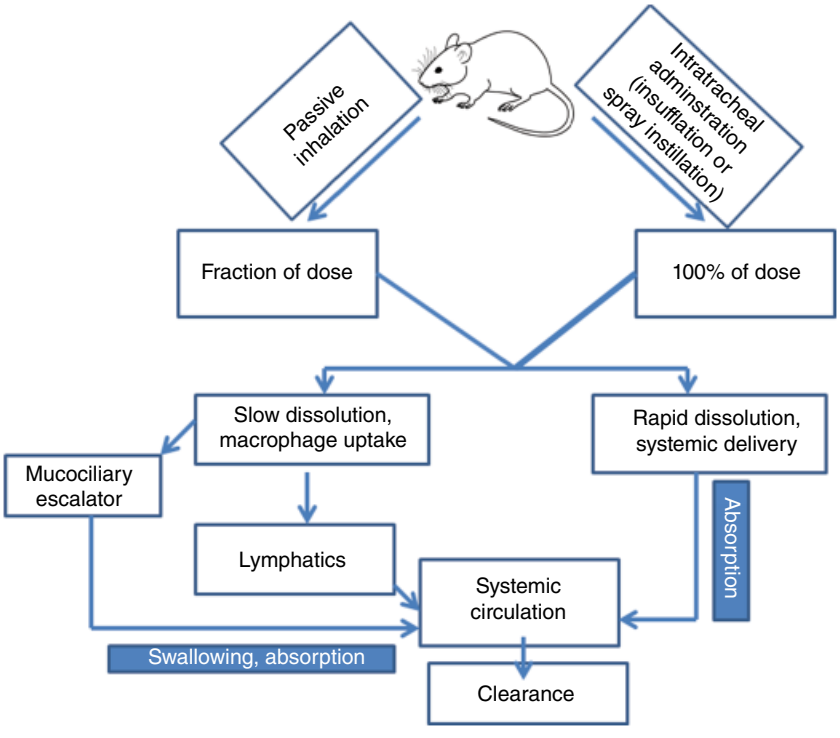


Figure 7.1 *Disposition of drugs after pulmonary administration in an animal model [36, 41, 45]*

drugs that have poor water solubility or poor oral bioavailability. Thus, it is usually assumed that once the drug dissolves in the lungs, it is rapidly absorbed [41]. Another approach designed to kill intracellular *Mycobacterium tuberculosis* (MTB) is to formulate anti-TB drugs as solid rigid inhalable particles that provide slow drug dissolution or controlled release of the drug so that they may be engulfed by alveolar macrophages [42, 43]. Thus, inhaled drug microparticles should reside in the alveoli long enough to be phagocytosed by these macrophages. Although macrophage targeting has shown promise in treating TB in animal models, it is still unclear if this approach alone is sufficient to eradicate bacteria in the lungs since there is evidence that MTB can exist both intra- and extra-cellularly. Hickey *et al*. recommended that if the pulmonary route is employed to treat TB, it should be supplemented with systemic therapy in order to treat pulmonary and extra-pulmonary TB [44].

Figure 7.1 illustrates the disposition of inhaled drug after pulmonary administration to an animal model. The fraction of the dose reaching the alveolar region varies with the method of delivery. Intratracheal delivery by either insufflation or spray instillation is an invasive method which requires that the animal is anaesthetized prior to intubation so that the entire dose is delivered into the lungs. Alternatively, drugs can be administered as aerosols by passive inhalation to animals that are awake but restrained for a short period of time so that they can passively inhale the aerosolized drug through their nose. Special chambers are designed to provide either nose-only or whole-body exposure of the animal to the aerosolized particles [36]. However, it should be noted that restraining the animal during

passive inhalation may cause an irregular, fast and shallow breathing pattern, which can influence the site of deposition of the particles along the respiratory tract.

The disposition of the drug after administration depends on its physicochemical properties, the formulation delivered and the site of deposition. Drugs administered as liquid aerosols are considered to be ready for absorption immediately after inhalation and thus they may have a short pulmonary half-life. Drugs with poor water solubility usually exhibit a long half-life and extended mean residence time in the lungs due to their slow rate of dissolution. If the deposited drug particles have sizes ranging from 1–3 microns and remain in the alveoli for an extended period, they can be phagocytosed by alveolar macrophages, then cleared through mucociliary clearance or carried to the lymphatic system. If the inhaled drug is orally bioavailable and administered to an animal that can breathe through its nose and mouth, then the amount reaching the systemic circulation is the sum of the orally and pulmonary absorbed fractions of the drug [45].

7.4 Pharmacokinetic Study Design

In order to design a successful PK study, each step in the study needs to be carefully considered and validated from the selection of the animal model to be used to the software and computational modeling approach employed in data analysis.

7.4.1 Animal Models

There are several animal models established for studying TB as a disease, including mice, guinea pigs, rabbits and non-human primates. Selection of an animal model for a preclinical PK or PK/PD study should be done by considering carefully the advantages and limitations of each model, but most importantly the differences in drug disposition and metabolism.

The mouse is the animal model used more frequently in TB studies because of its reduced cost and the availability of the immunological tools. The guinea pig model mimics the human immunological responses to TB and develops necrotic tubercular granuloma. The pathogenesis of TB in non-human primates closely resembles that in humans developing latent TB as well as necrosis and caseation. However, studies involving non-human primates are very expensive and require special veterinarians [46]. It is suggested that PK studies are first performed in healthy animals to avoid confounding factors inherent to the disease stage in infected animals and the degree of lung damage.

There are physiological and anatomical factors that need to be considered upon selection of a particular animal model, particularly in the case of pulmonary delivery. Small animal models such as mice and guinea pigs are obligate nose breathers while bigger animals such as dogs and monkeys can breathe through their nose and mouth, as can humans. Such a difference influences the fraction of the inhaled dose of an aerosolized drug administered by passive inhalation. For example, guinea pigs cannot inhale particles bigger than 3 μm due to the small cut-off diameter of their nose whereas humans can effectively inhale particles of aerodynamic diameter >10 μm. In addition, animal species have different values for respiratory parameters including differences in tidal volume, rate of breathing, and minute volume. All of these parameters in conjunction affect the extent of aerosol inhaled

and should be taken into consideration when calculating the dose of drug inhaled by passive inhalation. These factors are particularly crucial if the inhaled dose is to be used to determine its PK parameters accurately. Notably, differences in the anatomy and morphology of the respiratory tract such as the airway branching pattern, the angles of airway bifurcation sites, and the number of airway generations between different animal models will influence the site and extent of deposition of the inhaled drug in different regions of the respiratory tract, which in turn will heavily impact the pharmacokinetic behavior of the drug and its efficacy in the selected animal model [47].

A factor that is particularly important for anti-TB drugs that are subjected to extensive hepatic metabolism (Table 7.2) are the differences that exist in the enzymatic activity of the liver between animal species. These differences will affect their elimination rate, half-life, and consequently the duration of activity of these drugs in the body [48, 49]. Rifampicin is a well-known inducer for hepatic cytochrome P450 enzymes in mice; however, in rats and guinea pigs, the presence of rifampicin does not elicit changes in the hepatic enzyme levels [50].

Lastly, the efficacy of anti-TB drugs can significantly vary among different animal species. Rosenthal *et al*. evaluated the effect of replacing rifampicin with rifapentine in a regular TB treatment regimen in mice [51, 52]. In this study, rifapentine showed a higher sterilization activity in mice than did rifampicin, suggesting that replacing rifampicin by rifapentine would shorten the duration of TB treatment. However, studies performed by Dutta *et al*. using the same treatment in guinea pigs did not demonstrate the same effect [51, 52].

7.4.2 Biological Samples

The number of biological samples collected and the sampling time points are crucial for a successful PK study. To characterize systemic drug disposition, a sufficient number of blood samples must be collected so that the terminal (elimination) phase contains at least 4–5 time points. If there is an absorption phase, enough samples should be collected at the initial time points and around the time of maximum plasma concentration to accurately calculate the absorption rate constant. However, there must be an ethical and experimental balance when considering the numbers of samples that can be collected, the well-being of the animal and the cost and effort in sample analysis. PK data previously published from relevant studies and the physicochemical properties of the drug can help in the preliminary design of the PK study, taking into consideration differences in the dose, formulation, route of administration and the animal model selected. For drugs administered by the pulmonary route, frequent sampling at early time points is necessary since most drugs are rapidly absorbed from the lungs. Urine samples have been also used in PK studies; however, they cannot be collected as easily as can blood samples. This is further complicated by the fact that only a small fraction of the drug can be recovered from the urine if the drug is subject to high metabolic clearance.

Since the lungs are the main site of TB-infection, it is necessary to determine drug concentrations in the lung itself at different times to correlate them with drug distribution and elimination. Bronchoalveolar lavage (BAL) is usually performed to measure the fraction of the drug dose in the airways that remains to be absorbed into lung tissue. However, the BAL procedure requires special training to obtain the maximum amount of fluid and to avoid possible contamination with blood and injured epithelium during sampling.

Unfortunately, in small animal models BAL is a terminal procedure that can yield to only a one-time point sample. Therefore, to obtain a drug concentration versus time profile in the airways, several animals must be sacrificed for BAL sampling at each predetermined time point. The BAL fluid obtained typically contains drug in solution, or in particles and cells, mainly alveolar macrophages. Thus the BAL fluid obtained is usually centrifuged gently to separate the cell pellet. Drug concentrations are usually obtained by analyzing the BAL supernatant. If the BAL fluid is not centrifuged and sonicated to disrupt the content in the cells, the concentration of the drug in the BAL includes the amount of drug inside the alveolar macrophage cells and outside cells [53]. The drug concentration in the alveolar macrophages recovered from the BAL can be further determined by analyzing the drug concentration in the cell pellet by subtracting the concentration in BAL fluid before pellet disruption and after. For assessment of systemic drug distribution, organs such as liver, spleen and the lungs should be collected, and drug concentration should be measured in the corresponding tissue homogenate.

7.4.3 Analytical Method

The analytical method selected to determine drug concentration in biological samples must be validated according to Food and Drug Administration (FDA) guidelines or their equivalent in other parts of the world. Depending on the nature and composition of the biological sample, they may require different preparation prior to analysis including tissue homogenization and drug extraction from the biological matrix. The stability of the drug in the biological sample should be known as well as the effect of extraction procedures or sample processing such as oxidation, susceptibility to light, extreme pH, and higher temperatures. Sample handling and processing should be performed carefully to avoid sample or drug degradation. High-performance liquid chromatography (HPLC) methods are the most commonly used analytical methods to determine drug concentration in different types of samples, but other methods such as liquid chromatography–mass spectrometry (HPLC–MS) are used when higher sensitivity and specificity are required, but these methods tend to be more expensive than HPLC methods.

7.4.4 Calculation of PK Parameters

The simplest approach to determine drug disposition and to calculate its PK parameters is the non-compartmental analysis (NCA) method, as it does not require the assumption of a certain model as in the case of regression analysis. NCA can provide a quick comparison of drug disposition after administration by different routes. The initial estimates of certain PK parameters that are required for compartmental modeling analysis can be obtained by NCA. PK parameters calculated by NCA are the elimination rate constant (K_e), half-life ($t_{1/2}$), clearance (CL), volume of distribution (V_d), maximum drug concentration in plasma (C_{max}), time at which maximum drug concentration is obtained ($T_{max),}$ mean residence time (MRT) and the area under the plasma concentration versus time curve (AUC). K_e, C_{max} and T_{max} values can be determined directly from the plasma concentration versus time plot curves. The mathematical equations to obtain these and other PK parameters are well known and described elsewhere [54]. There are several computational software packages available for PK modeling but Phoenix WinNonlin, NONMEM and MATlab are the most popular in the United States.

The absolute bioavailability (F) is the PK parameter that is most commonly used to measure the systemic availability of a drug after administration by any route. It is calculated by the following equation:

$$F = \frac{\text{AUC}_{(\text{non-IV})}}{\text{AUC}_{\text{IV}}} \times \frac{\text{Dose}_{\text{IV}}}{\text{Dose}_{(\text{non-IV})}} \times 100 \tag{7.1}$$

In Equation (7.1) AUC_{IV} and $\text{AUC}_{(\text{non-IV})}$ are the AUC values after IV and non-IV administration, respectively. Dose_{IV} and $\text{Dose}_{\text{non-IV}}$ are the doses administered to the animal through IV and non-IV routes, respectively. In studies in which the drug is delivered by passive inhalation, it is difficult to determine the exact inhaled dose. In this case an approximate dose is calculated using an estimate of the aerosolized dose, the breathing rate and the tidal volume of the animal model. The inhaled dose of a drug can be calculated by the following equation:

$$\text{Dose deposited after passive inhalation} = \frac{C \times V_{\text{min}} \times T}{\text{BW}} \tag{7.2}$$

In Equation (7.2) C is the concentration of drug in the aerosol, V_{min} is the inhaled volume per minute, T is the duration of exposure to the aerosol and BW is the body weight [45].

In pulmonary delivery studies, 'F' can be used to determine the circulating drug concentration and the extent of drug elimination, particularly if the drug has negligible oral bioavailability.

If the PK study is performed to evaluate a new drug, a new formulation or a different route of administration, the disposition of this drug after IV administration needs to be included as a control because it is considered as the ultimate reference because drug bioavailability after IV administration is considered to be 100%. In addition, any significant differences between PK parameters ($t_{1/2}$, CL) of a drug after IV and non-IV administration can reveal either errors in the study or specific kinetic phenomena such as flip-flop kinetics. Normally, K_{a} is much higher than K_{e}, meaning that the absorption process of a drug is faster than the elimination process, which is the limiting step. In flip-flop kinetics the situation is 'flipped', where K_{a} is smaller than K_{e} because of the slow rate of absorption. The main evidence for flip-flop kinetics is established when the $t_{1/2}$ of a drug is longer than its $t_{1/2}$ after IV administration regardless of differences in the administered dose [55]. It is also important to rule out flip-flop kinetics to avoid overestimation of PK parameters, which may lead to false conclusions and negatively impact the outcome of an efficacy study with the anti-TB drug under investigation.

One of the reasons for poor drug bioavailability may be due to a slow rate of absorption. In this case, the mean absorption time (MAT) is a useful parameter to indicate the rate of absorption. MAT is calculated from Equation (7.3).

$$\text{MAT} = \text{MRT}_{(\text{non-IV})} - \text{MRT}_{\text{IV}} \tag{7.3}$$

The rate of drug absorption, K_{a}, can be calculated from the collected data by using the method of residuals or by compartment modeling. In general, compartmental modeling postulates that the body consists of different interconnecting compartments. Each of these compartments has a different volume of distribution and variable drug concentrations that are in equilibrium with each other based on the degree of drug distribution among these

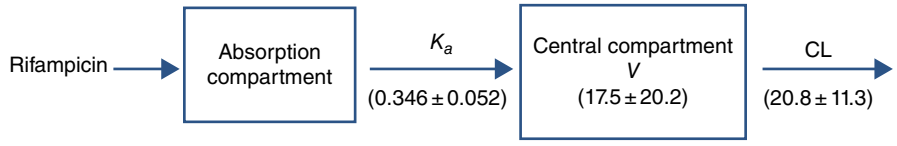


Figure 7.2 *A one-compartment model for rifampicin administered by oral route with first-order elimination. K_a, absorption rate constant; V, volume of distribution in the central compartment; CL, Clearance [56]*

compartments. The inter-compartmental constants estimated by compartmental modeling may provide more information on drug disposition. Different compartmental models can be proposed and the best model is selected based on how it fits the data visually (predicted data superimposes the observed data) and by the goodness-of-fit criteria [Akaike Criteria (AIC) and the weighted sum of squares residuals (WSSR)]. However, compartmental modeling is more complicated and time consuming than is NCA. Figure 7.2 illustrates a one-compartment model for rifampicin after oral administration to TB patients and the calculated PK parameters.

A more complicated type of compartmental model is the physiologically based pharmacokinetic model (PBPK) where the postulated compartments are real organs in the body such as brain, liver or kidney [57].

Deconvolution is a mathematical method used to characterize the input rate and cumulative amount of drug absorbed into systemic circulation [55]. Deconvolution analysis requires concentration–time data collected after extravascular and IV dosing [58]. This approach has not been used for anti-TB drugs but was used by Brindley *et al*. to calculate the rate and extent of absorption of fluticasone furoate from the lungs [59]. In this case, deconvolution PK analysis revealed that 90% of the drug was absorbed systemically in 20–30 hours depending on the formulation.

7.5 Implications of PK Parameters on Efficacy

Understanding the PK behavior of a drug is clinically useful to adjust the doses in individual patients and for therapeutic monitoring of drugs with narrow therapeutic index. However, PK studies are rarely used in the same way to monitor TB treatment. Thus, it may be possible that therapeutic failures, relapses and emergence of drug-resistant strains are the result of a lack of appropriate drug monitoring.

In our opinion, a clear understanding of PK parameters describing the absorption, distribution, metabolism and excretion for each anti-TB drug is invaluable to improve current therapies. In addition to the elements of a PK study discussed in previous sections, we offer some suggestions that have been useful in our group.

7.5.1 Tissue Samples

When drug concentrations are measured in specific organs, it is important to understand that this is only a single time point and that drug concentrations measured in this tissue are in equilibrium with those in plasma and that they will change as a function of time.

As mentioned previously, the rate of drug transfer between plasma and tissue, as well as the residence time of the drug in the tissue, are influenced by the physicochemical properties of the drug (Table 7.1), protein binding, and metabolism in the particular organ (Table 7.2).

Ideally, tissue samples would be collected at various time points in a similar manner as blood, to establish a tissue concentration versus time profile. However, as in the case of BAL collection, this procedure is terminal and the knowledge gained from such a study should be weighed against the number of animals required, and the ethical implications. It is also important to understand that the amount of drug in a homogenized tissue is the sum of the fraction of drug that exists, both intra- and extra-cellularly, in which the drug could be unevenly distributed [60]. Similarly, drug concentrations in plasma include both unbound drug and drug bound to plasma proteins. According to the current dogma, only the unbound fraction of the drug is considered to be pharmacologically active and can cross membrane barriers to reach the site of action in the lungs [61].

7.5.2 Pharmacokinetics of Anti-TB Drug in Granulomas

The tubercular granuloma that characterizes TB infection is an aggregation of different immune cells trapping the MTB in the middle, and is surrounded by a fibrous cuff. Particularly in the lungs, granulomas are low in blood supply, hypoxic and are most likely necrotic at the late stages of infection, making them highly impermeable to anti-TB drugs. Unless drugs are able to diffuse into the granuloma to kill the MTB, it is inconsequential that the drug has a favorable PK behavior in the plasma. Unfortunately, there is a scarcity of information about the diffusivity of anti-TB drugs inside the granuloma, mainly due to the technical difficulties encountered in isolating such lesions. Kjellsson *et al*. has modeled the distribution of a drug combination consisting of isoniazid, pyrazinamide, and moxifloxacin and rifampicin separately in the lung tissues and granulomas using a rabbit model. They reported that the concentrations these drugs with the exception of moxifloxacin were lower in the granuloma than were those in the plasma [62]. Figure 7.3 represents a

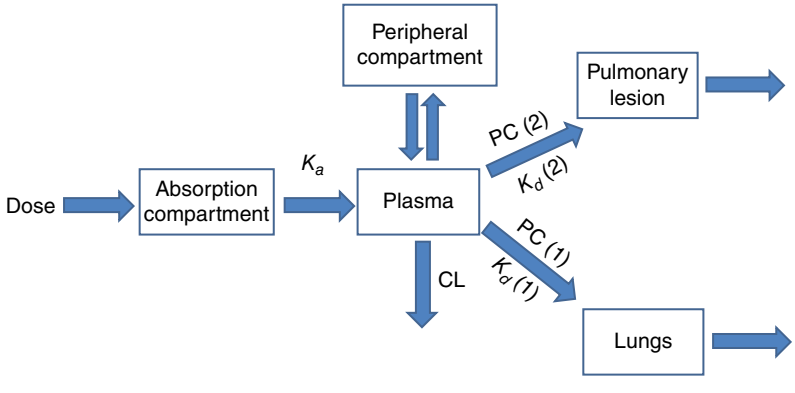


Figure 7.3 *A two-compartment model describing the absorption and distribution of pyrazinamide and the penetration coefficient of the drug in the lung and granulomas of TB. K_a, absorption rate constant; K_d (1) and K_d (2), distribution rate constant of pyrazinamide in the lungs and the pulmonary lesion, respectively; PC (1) and PC (2), penetration coefficient of pyrazinamide in the lungs and the pulmonary lesion, respectively; CL, clearance(adopted from [62])*

two-compartment model with first-order absorption and first-order elimination proposed by Kjellsson *et al*., and describes the distribution and penetration of pyrazinamide in the lung and granulomas.

7.5.3 PK/PD Correlations

The most convincing evidence of the anti-TB activity of a new molecule is the MIC. This PD parameter is defined as the minimum concentration of the drug that is sufficient to inhibit the growth of MTB after co-incubation over a period of time that depends on the method employed to determine it [63]. Measurement of MIC *in vitro* using only medium, bacteria and drug should be complemented by evaluating the ability of the drug candidate to kill MTB inside alveolar macrophages. Depending on the specific drug and its mechanism of action, it may be plausible that the extent of anti-TB activity *in vitro* would not translate in the same extent of *in vivo* effectiveness, despite the promising PK behavior. For this reason, the combination of PK and PD parameters (PK/PD) is considered to be the best predictor of antimicrobial effects. The most important PK/PD parameters are AUC/MIC, C_{max}/MIC and $T>$MIC defined as the duration at which the drug concentration in the plasma is higher than MIC. However, it should be noted that these combinations are different for each antibiotic class. For example, C_{max}/MIC is the PK/PD combination that best describes *in vivo* efficacy for aminoglycosides, whereas AUC/MIC predicts better the efficacy of flouroquinolones [64]. For anti-TB drugs, usually AUC/MIC and C_{max}/MIC correlate well with the antimicrobial activity of drugs that kill the bacteria intracellularly, such as rifamycins and fluoroquinolones. However, $T>$MIC, correlates better with drugs that act at the cell wall of the bacteria [61, 65]. The bactericidal activity of aerosolized rifampicin inhaled by TB-infected mice correlated best with the AUC/MIC PK/PD parameter [65].

7.6 Case Studies (Drugs Administered by Conventional and Pulmonary Routes)

Table 7.3 lists selected PK parameters obtained in different PK studies studying the disposition of anti-TB drugs in animal models and humans. The following case studies with rifampicin and capreomycin illustrate the importance of the factors discussed in this chapter.

7.6.1 Rifampicin

This first-line anti-TB drug is perhaps the one that has been more studied with respect to new formulations, its disposition (PK) in different species, and its efficacy alone or in selected combinations, or drug regimens. In the context of the present chapter, the majority of publications addressing the pharmacokinetics of an anti-TB drug and the influence of different factors on its disposition have been on rifampicin. It is mainly administered orally to patients and it's considered to be absorbed completely from the GI tract, although variations in absorption have been noted to be absorbed better in an acidic environment than in a basic or neutral environment [80]. Once it is absorbed, rifampicin is widely distributed but it is reported to be 80–89% bound to plasma proteins [18] (Table 7.2), although this binding may be easily reversible. Most notably, it acts as both inducer and substrate of

Table 7.3 *PK parameters of anti-TB drugs after their administration by the conventional and pulmonary routes to different species*

Drug	Species	Delivery route	Dose	t_{max} (h)	C_{max} (μg/ml)	$t_{1/2}$ (h)	Bioavailability	Ref.
Isoniazid	Human	Oral	300 mg	5.53 ± 2.9	1.02 ± 1.1	2.9	Estimated to be 1	[66]
	Mouse	Oral	25 mg/kg, once	—	28.2 ± 3.8	1.7 ± 0.2	—	[67]
	Rat	Oral	3 mg/kg, multiple	—	0.31	2.65	—	[68]
			30 mg/kg, multiple	—	8.38	1.04	—	
	Guinea pig	Oral	10 mg/kg	2.00 ± 0.0	1.71 ± 0.3	3.50 ± 0.7	0.58	[69]
Rifampicin	Human	Oral	600 mg	10.54 ± 3.2	2.42 ± 1.3	3.95 (2.3–6.0)	Estimated to be 1	[70]
	Mouse	Oral	10 mg/kg	0.5	18.5	4.4	—	[71]
	Rat	Oral	3 and 30 mg/kg	—	0.15, 8.69	4.41, 2.44	—	[68]
	Guinea pig	Oral	2.5 mg/kg	1.00 ± 0.5	0.08 ± 0.02	2.63 ± 1.16	0.17 ± 0.05	[39]
	Rat	Pulmonary (INS)[a]	600 μg (2.06 mg/kg)	1–2	0.67–1.3	—	—	[34]
	Guinea pig	Pulmonary (INS)[a]	2.5 mg/kg	0.19 ± 0.1	0.6 ± 0.1	2.4 ± 1.6	0.7 ± 0.2	[39]
Pyrazinamide	Rat	Oral	300 mg/kg	1.04	218.6	—	—	[68]
	Guinea pig	Oral	25 mg/kg	2.00 ± 0.0	23.80 ± 2.1	5.30 ± 0.6	0.92	[69]
	Guinea pig	IV	25 mg/kg	0.17 ± 0.0	71.10 ± 4.0	4.95 ± 0.7	—	
	Human	Oral	30 mg/kg	53.4 (±10.4)	1.43 (± 1.06)	8.51 (5.4–10.2)	Estimated to be 1	[72]
	Guinea pig	Pulmonary (INS)[a]	5 mg/kg	0.08 ± 0.0	6.8 ± 1.0	2.13 ± 0.8	Estimated to be 1	[b]
Ethambutol	Human	Oral	25 mg/kg	4.5 ± 1.0	2.5 ± 0.9	$t_{1/2\alpha}$ 1.3 and $t_{1/2\beta}$ 12.4	Estimated to be 1	[73]
	Rat	Oral	300 mg/kg	30.04	—	2.90	—	[68]

(*continued*)

Table 7.3 *(Continued)*

Drug	Species	Delivery route	Dose	t_{max} (h)	C_{max} (μg/ml)	$t_{1/2}$ (h)	Bioavailability	Ref.
Ethionamide	Human	Oral	500 mg	2.3 (0.99–6.1)	1.7 (0.8–3.0)	1.8 (37%)	—	[74]
	Guinea pig	Pulmonary (INS)[a]	6 mg/kg	0.08 ± 0.0	3.71 ± 0.6	0.75 ± 0.6	0.85 ± 0.1	[c]
Clofazimine	Mice	Oral	25 mg/kg	1–4	0.43	87.46	—	[75]
PA-824	Guinea pig	Pulmonary (INS)[a]	20, 40 and 60 mg/kg	4.3 ± 1.0, 3.3 ± 2.1, 3.6 ± 2.9	2.0 ± 0.6, 3.4 ± 1.1, 4.6 ± 2.5	2.8 ± 0.1, 4.4 ± 1.0, 5.9 ± 2.5	0.59 ± 0.2, 0.63 ± 0.3, 0.62 ± 0.1	[38]
		Oral	40 mg/kg, once	4.00 ± 0.63	4.14 ± 0.78	2.43 ± 0.56	0.56 ± 0.12	
	Human	Oral	50–1500, once	4–5	3–3.8	16–20	—	[76]
Capreomycin	Guinea pig	IV	20 mg/kg	0.08 ± 0.0	53.56 ± 3.9	0.75 ± 0.1	1.0 ± 0.0	[77]
		IM	20 mg/kg	1.09 ± 0.3	32.33 ± 3.8	1.09 ± 0.3	1.2 ± 0.2	
		Pulmonary (INS)[a]	1.4, 7.2 and 14.5 mg/kg	0.5 ± 0.3, 0.38 ± 0.1, 0.31 ± 0.1	0.92 ± 0.4, 3.34 ± 0.5, 6.7 ± 1.3	1.22 ± 0.6, 1.68 ± 0.4, 1.53 ± 0.4	0.41 ± 0.1, 0.59 ± 0.1, 0.54 ± 0.1	
	Human	Pulmonary	25, 50, 150 and 300 mg	2.4–2.8	0.169–2.3	NC, 4.2, 4.4 and 4.8	—	[78]
PAS	Rat	Pulmonary (INS)[a]	5 mg	0–0.5	11 ± 1.0	—	—	[79]

[a] (INS): intratracheal insufflation. NC: Not calculated.
[b] Garcia-Contreras, L. (2014) Unpublished results. Oklahoma City, OK, University of Oklahoma Health Science Center.
[c] Garcia-Contreras, L. (2008) Unpublished results. Chapel Hill, NC, University of North Carolina.

metabolic enzymes and P-glycoprotein, and efflux pump in the membrane of several tissues [81]. Remarkably, rifampicin auto-induces its metabolism, which causes the decrease of its resulting plasma concentration and half-life after repeated administration [17].

The effect of formulation on drug disposition can be illustrated by the PK studies performed with rifampicin. A review of the literature for the past 10 years revealed that rifampicin is one of the most commonly formulated anti-TB drugs for pulmonary delivery. Coowanitwong *et al*. [34] compared the disposition of rifampicin after pulmonary administration of slow-release rifampicin microparticles composed of different ratios of the drug to polylactic acid (PLA) and poly(lactic-co-glycolic acid) (PLGA polymers) to Sprague Dawley rats. The C_{max} values obtained after pulmonary administration of slow-release rifampicin polymeric microparticles (0.67–1.3 μg/ml) were significantly lower than those obtained after insufflation of polymer-free rifampicin microparticles (6.97 μg/ml) owing to the slow absorption of rifampicin from the slow-release particles.

Pharmacokinetic studies performed by de Steenwinkel *et al*. evaluated the disposition of two rifampicin doses (10 and 160 mg/kg) after oral administration to female BALB/C mice and the influence of concomitant administration of isoniazid and pyrazinamide [71]. As expected, when rifampicin was administered alone, AUC, C_{max} and $t_{1/2}$ increased proportionally with the dose and these parameters did not change significantly when rifampicin was co-administered with isoniazid and pyrazinamide. Kumar *et al*. evaluated the disposition of rifampicin in TB-infected, male Wistar rats that had been receiving daily doses of 30 mg/kg rifampicin for 18 consecutive days [68]. Comparing the studies by de Steenwinkel *et al*. with those of Kumar *et al*., in which the doses were 3-fold higher (10 mg/kg vs. 30 mg/kg), C_{max} and AUC are much larger in mice (18.5 μg/ml and 125.1 μg.h/ml, respectively) than in rats (8.69 μg/ml and 54.71 μg.h/ml, respectively). This is surprising since given that besides the larger dose, mice have faster metabolism than do rats and thus one would expect a smaller C_{max} and AUC in mice than in rats. A plausible explanation in this difference may be that mice were healthy whereas rats were TB-infected; however, it is unlikely that this was a decisive factor as the drug was given orally. Perhaps a better explanation for the smaller C_{max} and AUC in rats is the auto-induction of its metabolism that is known to occur after repeated administrations of rifampicin, whereas the PK parameters in mice were calculated after a single-dose administration. However, a puzzling difference arises when comparing the disposition of rifampicin after oral administration in mice and in guinea pigs. Sung *et al*. evaluated the disposition of rifampicin by the oral route as a suspension and by the pulmonary route as micro- and nano-particles [39]. When correcting the C_{max} obtained in mice by the dose (18.5 μg/ml//10 mg/kg), this was 50-fold larger than in the guinea pig (0.08 μg/ml//2.5 mg/kg), even though they were both single-dose studies. This illustrates the different disposition of this drug in different species as well as the effect of multiple- versus single-dose studies with rifampicin.

The effect of the route of drug administration also has important implications in the disposition of rifampicin. Coowanitwong *et al*. studied the disposition of rifampicin after pulmonary administration of 2 mg/kg to male Sprague Dawley rats [34]. Comparing the PK parameters in this study with those of Kumar *et al*. [68] that employed a similar dose but by the oral route (3 mg/kg), the C_{max} obtained by Coowanitwong *et al*. is 46-fold larger and the $t_{1/2}$ 4.5-fold longer (6.97 μg/ml, and 4.4 h) than those reported by Kumar *et al*. (0.15 μg/ml, and 0.99 h). Despite the differences in route of administration (pulmonary

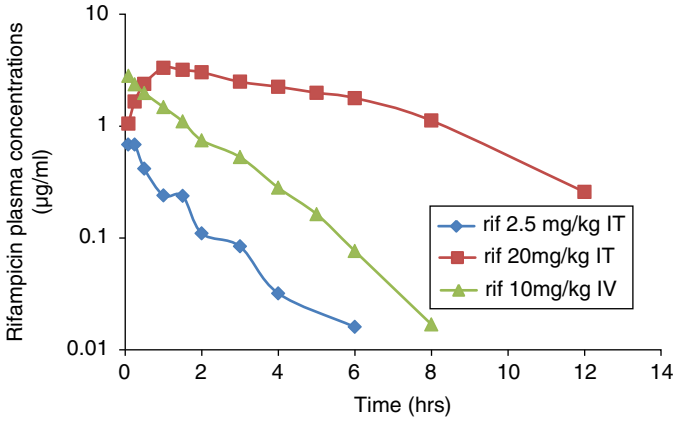


Figure 7.4 *Plasma concentrations versus time of rifampicin after intratracheal insufflation of 2.5 and 20 mg/kg and IV administration of 10 mg/kg in guinea pigs (Log scale). IT, intratracheal insufflation [39, 82]*

vs. oral) and the rat strain (Sprague Dawley vs. Wistar), perhaps the main reason for these large differences is the auto-metabolism of rifampicin after repeated administration in the study by Kumar *et al.*

Another study that illustrates the effect of route of administration, formulation and dose was the evaluation of rifampicin disposition in the guinea pig [82]. In this study, the drug was administered as porous particles at a dose of 20 mg/kg by insufflation and compared with the disposition after IV administration of a rifampicin solution at a dose of 10 mg/kg. Figure 7.4 combines the plasma concentration versus time plots of this study and the one performed by Sung *et al.* [39]. The C_{max} and t_{max} values obtained after insufflation of the 20 mg/kg dose of rifampicin as porous particles were approximately 6 times higher (3.50 µg/ml) than their corresponding values obtained after insufflation of the nanoparticles at a smaller dose (2.5 mg/kg) employed in the study by Sung *et al.* (0.67 µg/ml) [39]. The differences in dose (8-fold) didn't account for the differences in the magnitude of C_{max} (6-fold). The explanation for this difference can be found by comparing the terminal slope of the plasma concentration versus time plots of the 20 mg/kg insufflated dose with that after IV administration (Figure 7.4). This comparison revealed the occurrence of flip-flop kinetic phenomenon which could be attributed to the low aqueous solubility of rifampicin (Table 7.1) and the limited fluid available for dissolution in the lungs. Thus, this illustrates the importance of having an IV group in a PK study design.

Unfortunately, there are no studies with inhaled rifampicin in humans, but there are several reports of its disposition after oral administration. Peloquin *et al.* studied the effect of food on the disposition of rifampicin in healthy male and female volunteers [70]. Oral administration of rifampicin with food prolonged the T_{max} from 2.43 h in fasting patients to 4.4 h in patients that had eaten a breakfast with a high fat content. In this case, it is possible that the presence of food delayed the absorption of rifampicin from the intestinal wall, resulting in this 2-fold increase in *T*max. In the same manner, this may be the reason for the significantly lower C_{max} observed in fed patients (7.27 µg/ml) compared with fasting subjects (10.54 µg/ml).

7.6.2 Capreomycin

The pharmacokinetics of capreomycin, a second-line anti-TB agent, has been studied in mice, rats, guinea pigs and humans by parenteral routes (IV, SC, IM) and by the pulmonary route, in either solution, liposomal suspension or powders for inhalation.

Le Conte *et al*. administered capreomycin intravenously as a solution or liposomal formulation to C59 Bulb mice at a dose of 120 mg/kg [28]. The liposomal formulation extended 2 fold the $t_{1/2}$ of capreomycin (from 0.18 ± 0.05 to 0.4 ± 0.05 h) and increased the AUC from 34 ± 6.4 to 50.4 ± 8.8 μg.h/ml, demonstrating that specialized formulations can modify significantly the systemic PK of this drug. Moreover, capreomycin concentrations were detected for 6 h in lung tissue after IV administration of the free and liposomal drug with the liposomal formulation increasing 1.6 times the capreomycin concentration in lung tissue compared with that after IV administration of free drug.

Seventeen years later, Reisfield *et al*. performed PK studies in the same strain, gender and age of mice using a capreomycin solution injected subcutaneously at 100 and 250 mg/kg [57]. Interestingly, mice in the Reisfield study exhibited much lower plasma concentrations at 0.5 h (60 and 170 ng/ml, respectively) [57] than those reported by Le Conte at the same time for either formulation (41–42 μg/ml) [28]. In addition, capreomycin concentrations were detected until 2 h in lung tissue in the Reisfield study, whereas, in the Le Conte study, capreomycin concentrations after injection of free or liposomal drug were detectable up until 6 h [28, 57]. These large differences between the two studies using the same animal model and similar doses may be due to other differences in factors of each study, such as the source of the drug, the site of injection and the analytical method employed. Capreomycin in the Le Conte study was of pharmaceutical grade (Capastat, Eli Lilly) whereas the capreomycin in the Reisfield study was reagent grade (Sigma Chemical Co.). Lee *et al*. and Garcia-Contreras *et al*. have also observed differences in the PK parameters of capreomycin when using drugs from different sources [83, 84]. Another difference between Le Conte and Reisfield studies is the site of injection; whereas Le Conte *et al*. injected capreomycin intravenously, Reisfield *et al*. injected it subcutaneously. Perhaps the most significant difference between these studies is the analytical method employed to determine the drug concentrations in the biological samples. Le Conte *et al*. assayed capreomycin by a microbiological method in AMS agar medium using *Bacillus subtilis* as the bacterial indicator whereas Reisfield *et al*. employed liquid chromatography–tandem mass spectrometry (LC/MS/MS), which is the most sophisticated and sensitive method. Therefore, the comparison between these studies illustrates the importance of selection of the source, purity and quality of the drug as well as the analytical method that is used to analyze the drug concentrations in biological fluids.

As mentioned in previous sections, it is important to consider the species selected for the PK study as it may have important implications in the magnitude of the PK parameters obtained. This factor can be illustrated by comparing the $t_{1/2}$ of capreomycin after IV injection. For example, the $t_{1/2}$ of capreomycin in mice is 0.18–0.4 h compared to 1.0–1.1 h and 0.75 h in rats and guinea pigs, respectively. These differences in $t_{1/2}$ may be due to metabolic differences between mice and rats or guinea pigs.

The effect of route of administration, using the same animal model, is illustrated in the following example [77]. Capreomycin was administered to guinea pigs by the IV, IM or

pulmonary routes as solution or powders. Whereas the AUC was significantly larger after IV and IM administration of capreomycin solution (49.29 and 58.2 μg.h/ml, respectively) than after pulmonary administration of powders (16.95 μg.h/ml), $t_{1/2}$ was significantly longer after pulmonary administration (1.53 h) than after IV or IM injections (0.75 and 1.09 h, respectively). Besides the differences in the routes of administration, it is also possible that this difference in $t_{1/2}$ is also due to the different formulations (solution versus powders) as well as the time needed to dissolve the powder and its rate of dissolution in the lung environment.

With respect to pulmonary delivery, perhaps the most relevant comparison is that of animal models to humans. In a phase 1 clinical study, escalating doses of capreomycin powder for inhalation were administered to healthy subjects using a hand-held inhaler [80]. The highest dose given to humans (3.77 mg/kg) was in between the low and high doses given to guinea pigs (1.4 and 7.2 mg/kg) [77]. The dose in humans resulted in a similar C_{max} (2.14 μg/ml//3.77 mg/kg) compared with that in guinea pigs when corrected by dose (3.34 μg/ml//7.2 mg/kg). However, T_{max} was about 7 times longer in humans than in guinea pigs (2.8 h versus 0.38 h) and the $t_{1/2}$ was almost 3 times as long (4.8 h in humans versus 1.68 h in guinea pigs) [77, 78]. In addition to the significant difference in size between humans and guinea pigs, other factors may have played a role in these differences, such as the method of pulmonary delivery (passive inhalation versus insufflations), the alveolar area available for dissolution and the metabolic rate between these two species.

References

[1] WHO The Stop TB Strategy (2006). Building on and enhancing DOTS to meet the TB-related Millennium Development Goals 2006. Available from: WHO/HTM/TB/2006.368.
[2] Hiruy, H., Rogers, Z., Mbowane, C. *et al*. (2015) Subtherapeutic concentrations of first-line anti-TB drugs in South African children treated according to current guidelines: the PHATISA study. *J. Antimicrob. Chemother.*, **70**(4), 1115–1123.
[3] Isoniazid (2008). *Tuberculosis (Edinburgh)*, **88**(2), 112–116.
[4] Rifampin (2008). *Tuberculosis (Edinburgh)*, **88**(2), 151–154.
[5] Pyrazinamide (2008). *Tuberculosis (Edinburgh)*, **88**(2), 141–144.
[6] Ethambutol (2008). *Tuberculosis (Edinburgh)*, **88**(2), 102–105.
[7] Girgis, E.H., Gad, Z. and Maram, M. (1974) Ion-pair extraction and precipitation methods for ethambutol determination. *J. Pharm. Sci.*, **63**(11), 1764–1767.
[8] Ethionamide (2008). *Tuberculosis (Edinburgh)*, **88**(2), 106–108.
[9] Para-aminosalicylic acid (2008). *Tuberculosis (Edinburgh)*, **88**(2), 137–138.
[10] Kanamycin (2008). *Tuberculosis (Edinburgh)*, **88**(2), 117–118.
[11] Capreomycin (2008). *Tuberculosis (Edinburgh)*, **88**(2), 89–91.
[12] Levofloxacin (2008). *Tuberculosis (Edinburgh)*, **88**(2), 119–121.
[13] Clofazimine (2008). *Tuberculosis (Edinburgh)*, **88**(2), 96–99.
[14] Rifapentine (2008). *Tuberculosis (Edinburgh)*, **88**(2), 155–158.
[15] Rifabutin (2008). *Tuberculosis (Edinburgh)*, **88**(2), 145–147.
[16] WHO Prequalification of Medicines Programme (2009). Biopharmaceutics Classification System (BCS)-based biowaver applications: anti-tuberculosis medicines. World Health Organization, Geneva, Switzerland.
[17] Holdiness, M.R. (1984) Clinical pharmacokinetics of the antituberculosis drugs. *Clin. Pharmacokinet.*, **9**(6), 511–544.
[18] Acocella, G. (1978) Clinical pharmacokinetics of rifampicin. *Clin. Pharmacokinet.*, **3**(2), 108–127.

[19] Loos, U., Musch, E., Jensen, J.C. *et al*. (1985) Pharmacokinetics of oral and intravenous rifampicin during chronic administration. *Klin. Wochenschr.*, **63**(23), 1205–1211.
[20] Stamatakis, G., Montes, C., Trouvin, J.H. *et al*. (1988) Pyrazinamide and pyrazinoic acid pharmacokinetics in patients with chronic renal failure. *Clin. Nephrol.*, **30**(4), 230–234.
[21] Jonsson, S., Davidse, A., Wilkins, J. *et al*. (2011) Population pharmacokinetics of ethambutol in South African tuberculosis patients. *Antimicrob. Agents Chemother.*, **55**(9), 4230–4237.
[22] Pan, X., Wang, L., Grundemann, D. and Sweet, D.H. (2013) Interaction of Ethambutol with human organic cation transporters of the SLC22 family indicates potential for drug-drug interactions during antituberculosis therapy. *Antimicrob. Agents Chemother.*, **57**(10), 5053–5059.
[23] Boxer, G.E., Jelinek, V.C. and Edison, A.O. (1949) Streptomycin; clearance and binding to protein. *J. Pharmacol. Exp. Ther.*, **97**(1), 93–104.
[24] Gordon, R.C., Regamey, C. and Kirby, W.M. (1972) Serum protein binding of the aminoglycoside antibiotics. *Antimicrob. Agents Chemother.*, **2**(3), 214–216.
[25] Zhu, M., Burman, W.J., Jaresko, G.S. *et al*. (2001) Population pharmacokinetics of intravenous and intramuscular streptomycin in patients with tuberculosis. *Pharmacotherapy*, **21**(9), 1037–1045.
[26] Dover, L.G., Corsino, P.E., Daniels, I.R. *et al*. (2004) Crystal structure of the TetR/CamR family repressor Mycobacterium tuberculosis EthR implicated in ethionamide resistance. *J. Mol. Biol.*, **340**(5), 1095–1105.
[27] Johnson, D.A. and Smith, K.D. (2006) The efficacy of certain anti-tuberculosis drugs is affected by binding to alpha-1-acid glycoprotein. *Biomed. Chromatogr.*, **20**(6–7), 551-–60.
[28] Le Conte, P., Le Gallou, F., Potel, G. *et al*. (1994) Pharmacokinetics, toxicity, and efficacy of liposomal capreomycin in disseminated Mycobacterium avium beige mouse model. *Antimicrob. Agents Chemother.*, **38**(12), 2695–2701.
[29] Moxifloxacin (2008) *Tuberculosis (Edinburgh)*, **88**(2), 127–131.
[30] Cholo, M.C., Steel, H.C., Fourie, P.B. *et al*. (2012) Clofazimine: current status and future prospects. *J. Antimicrob. Chemother.*, **67**(2), 290–298.
[31] Peloquin, C.A., Henshaw, T.L., Huitt, G.A. *et al*. (1994) Pharmacokinetic evaluation of para-aminosalicylic acid granules. *Pharmacotherapy*, **14**(1), 40–46.
[32] Raviglione, M.C., Narain, J.P. and Kochi, A. (1992) HIV-associated tuberculosis in developing countries: clinical features, diagnosis, and treatment. *Bull. World Health Organ.*, **70**(4), 515–526.
[33] Gurumurthy, P., Ramachandran, G., Hemanth Kumar, A.K. *et al*. (2004) Decreased bioavailability of rifampin and other antituberculosis drugs in patients with advanced human immunodeficiency virus disease. *Antimicrob. Agents Chemother.*, **48**(11), 4473–4475.
[34] Coowanitwong, I., Arya, V., Kulvanich, P. and Hochhaus, G. (2008) Slow release formulations of inhaled rifampin. *AAPS J.*, **10**(2), 342–348.
[35] Fiegel, J., Garcia-Contreras, L., Thomas, M. *et al*. (2008) Preparation and in vivo evaluation of a dry powder for inhalation of capreomycin. *Pharm. Res.*, **25**(4), 805–811.
[36] Misra, A., Hickey, A.J., Rossi, C. *et al*. (2011) Inhaled drug therapy for treatment of tuberculosis. *Tuberculosis (Edinburgh)*, **91**(1), 71–81.
[37] Pandey, R., Sharma, S. and Khuller, G.K. (2004) Nebulization of liposome encapsulated antitubercular drugs in guinea pigs. *Int. J. Antimicrob. Agents*, **24**(1), 93–94.
[38] Sung, J.C., Garcia-Contreras, L., Verberkmoes, J.L. *et al*. (2009) Dry powder nitroimidazopyran antibiotic PA-824 aerosol for inhalation. *Antimicrob. Agents Chemother.*, **53**(4), 1338–1343.
[39] Sung, J.C, Padilla, D.J., Garcia-Contreras, L. *et al*. (2009) Formulation and pharmacokinetics of self-assembled rifampicin nanoparticle systems for pulmonary delivery. *Pharm. Res.*, **26**(8), 1847–1855.
[40] Olsson, B., Bondeson, E., Borgström, L. *et al*. (2011) Pulmonary Drug Metabolism, Clearance, and Absorption. In: Hickey, A.J. and Smyth, H.D.C. (eds) Controlled Pulmonary Drug Delivery, 21, *Advances in Delivery Science and Technology*. Springer Science+Business Media, New York, NY, pp. 21–50.
[41] Issar, M.M.C., Khan, P. and Hochhaus, G. (2004) Pharmacokinetics and pharmacodynamics of drugs delivered to the lungs. In: Hickey, A.J. (ed.) *Pharmaceutical Inhalation Aerosol Technology*. Marcel Dekker, Inc., New York, NY, pp. 215–252.

[42] Maretti, E., Rossi, T., Bondi, M. *et al*. (2014) Inhaled solid lipid microparticles to target alveolar macrophages for tuberculosis. *Int. J. Pharm.*, **462**(1–2), 74–82.
[43] Parikh, R., Dalwadi, S., Aboti, P. and Patel, L. (2014) Inhaled microparticles of antitubercular antibiotic for in vitro and in vivo alveolar macrophage targeting and activation of phagocytosis. *J. Antibiot. (Tokyo)*, **67**(5), 387–394.
[44] Hickey, A.J., Misra, A. and Fourie, P.B. (2013) Dry powder antibiotic aerosol product development: inhaled therapy for tuberculosis. *J. Pharm. Sci.*, **102**(11), 3900–3907.
[45] Sakagami, M. (2006) In vivo, in vitro and ex vivo models to assess pulmonary absorption and disposition of inhaled therapeutics for systemic delivery. *Adv. Drug Deliv. Rev.*, **58**(9–10), 1030–1060.
[46] Guirado, E. and Schlesinger, L.S. (2013) Modeling the Mycobacterium tuberculosis Granuloma - the Critical Battlefield in Host Immunity and Disease. *Front. Immunol.*, **4**, 98.
[47] Garcia-Contreras, L. (2011) In vivo animal models for controlled-release pulmonary drug delivery. In: Smyth, H.D.C. and Hickey A.J. (eds) *Controlled Pulmonary Drug Delivery*. Springer Science+Business Media, LLC, New York, NY, pp. 443–475.
[48] Glinsukon, T., Benjamin, T., Grantham, P.H. *et al*. (1975) Enzymic N-acetylation of 2,4-toluenediamine by liver cytosols from various species. *Xenobiotica*, **5**(8), 475–483.
[49] Ishizuka, H., Konno, K., Shiina, T. *et al*. (1999) Species differences in the transport activity for organic anions across the bile canalicular membrane. *J. Pharmacol. Exp. Ther.*, **290**(3), 1324–1330.
[50] Strolin Benedetti, M. and Dostert, P. (1994) Induction and autoinduction properties of rifamycin derivatives: a review of animal and human studies. *Environ. Health Perspect.*, **102** Suppl. 9, 101–105.
[51] Dutta, N.K., Illei, P.B., Peloquin, C.A. *et al*. (2012) Rifapentine is not more active than rifampin against chronic tuberculosis in guinea pigs. *Antimicrob. Agents Chemother.*, **56**(7), 3726–3731.
[52] Rosenthal, I.M., Tasneen, R., Peloquin, C.A. *et al*. (2012) Dose-ranging comparison of rifampin and rifapentine in two pathologically distinct murine models of tuberculosis. *Antimicrob. Agents Chemother.*, **56**(8), 4331–4340.
[53] Doan, T.V., Gregoire, N., Lamarche, I. *et al*. (2013) A preclinical pharmacokinetic modeling approach to the biopharmaceutical characterization of immediate and microsphere-based sustained release pulmonary formulations of rifampicin. *Eur. J. Pharm. Sci.*, **48**(1–2), 223–230.
[54] Gabrielsson, J. and Weiner, D. (2012) Non-compartmental analysis. *Methods Mol. Biol*., **929**, 377–389.
[55] Yanez, J.A., Remsberg, C.M., Sayre, C.L. *et al*. (2011) Flip-flop pharmacokinetics—delivering a reversal of disposition: challenges and opportunities during drug development. *Ther. Deliv.*, **2**(5), 643–672.
[56] Pasipanodya, J.G., McIlleron, H., Burger, A. *et al*. (2013) Serum drug concentrations predictive of pulmonary tuberculosis outcomes. *J. Infect. Dis.*, **208**(9), 1464–1473.
[57] Reisfeld, B., Metzler, C.P., Lyons, M.A. *et al*. (2012) A physiologically based pharmacokinetic model for capreomycin. *Antimicrob. Agents Chemother.*, **56**(2), 926–934.
[58] Allen, A., Bareille, P.J. and Rousell, V.M. (2013) Fluticasone furoate, a novel inhaled corticosteroid, demonstrates prolonged lung absorption kinetics in man compared with inhaled fluticasone propionate. *Clin. Pharmacokinet.*, **52**(1), 37–42.
[59] Brindley, C., Falcoz, C., Mackie, A.E. and Bye, A. (2000) Absorption kinetics after inhalation of fluticasone propionate via the Diskhaler, Diskus and metered-dose inhaler in healthy volunteers. *Clin. Pharmacokinet*., **39** Suppl. 1, 1–8.
[60] Mouton, J.W., Theuretzbacher, U., Craig, W.A. *et al*. (2008) Tissue concentrations: do we ever learn? *J. Antimicrob. Chemother.*, **61**(2), 235–237.
[61] Budha, N.R., Lee, R.E. and Meibohm, B. (2008) Biopharmaceutics, pharmacokinetics and pharmacodynamics of antituberculosis drugs. *Curr. Med. Chem.*, **15**(8), 809–825.
[62] Kjellsson, M.C., Via, L.E., Goh, A. *et al*. (2012) Pharmacokinetic evaluation of the penetration of antituberculosis agents in rabbit pulmonary lesions. *Antimicrob. Agents Chemother.*, **56**(1), 446–457.
[63] Andrews, J.M. (2001) Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother.*, **48** Suppl 1, 5–16.

[64] Wright, D.H., Brown, G.H., Peterson, M.L. and Rotschafer, J.C. (2000) Application of fluoroquinolone pharmacodynamics. *J. Antimicrob. Chemother.*, **46**(5), 669–683.
[65] Jayaram, R., Gaonkar, S., Kaur, P. *et al*. (2003) Pharmacokinetics-pharmacodynamics of rifampin in an aerosol infection model of tuberculosis. *Antimicrob. Agents Chemother.*, **47**(7), 2118–2124.
[66] Peloquin, C.A., Namdar, R., Dodge, A.A. and Nix, D.E. (1999) Pharmacokinetics of isoniazid under fasting conditions, with food, and with antacids. *Int. J. Tuberc. Lung Dis.*, **3**(8), 703–710.
[67] Handbook of anti-tuberculosis agents (2008) Introduction. *Tuberculosis (Edinburgh)*, **88**(2), 85–86.
[68] Kumar, N., Vishwas, K.G., Kumar, M. *et al*. (2014) Pharmacokinetics and dose response of anti-TB drugs in rat infection model of tuberculosis. *Tuberculosis (Edinburgh)*, **94**(3), 282–286.
[69] Pandey, R., Sharma, A., Zahoor, A. *et al*. (2003) Poly (DL-lactide-co-glycolide) nanoparticle-based inhalable sustained drug delivery system for experimental tuberculosis. *J. Antimicrob. Chemother.*, **52**(6), 981–986.
[70] Peloquin, C.A., Namdar, R., Singleton, M.D. and Nix, D.E. (1999) Pharmacokinetics of rifampin under fasting conditions, with food, and with antacids. *Chest*, **115**(1), 12–18.
[71] de Steenwinkel, J.E., Aarnoutse, R.E., de Knegt, G.J. *et al*. (2013) Optimization of the rifampin dosage to improve the therapeutic efficacy in tuberculosis treatment using a murine model. *Am. J. Respir. Crit. Care Med.*, **187**(10), 1127–1134.
[72] Peloquin, C.A., Bulpitt, A.E., Jaresko, G.S. *et al*. (1998) Pharmacokinetics of pyrazinamide under fasting conditions, with food, and with antacids. *Pharmacotherapy*, **18**(6), 1205–1211.
[73] Peloquin, C.A., Bulpitt, A.E., Jaresko, G.S. *et al*. (1999) Pharmacokinetics of ethambutol under fasting conditions, with food, and with antacids. *Antimicrob. Agents Chemother.*, **43**(3), 568–572.
[74] Auclair, B., Nix, D.E., Adam, R.D. *et al*. (2001) Pharmacokinetics of ethionamide administered under fasting conditions or with orange juice, food, or antacids. *Antimicrob. Agents Chemother.*, **45**(3), 810–814.
[75] Swanson, R.V., Adamson, J., Moodley, C. *et al*. (2015) Pharmacokinetics and pharmacodynamics of clofazimine in the mouse model of tuberculosis. *Antimicrob. Agents Chemother.* **59**(6), 3042–3051.
[76] Ginsberg, A.M., Laurenzi, M.W., Rouse, D.J. *et al*. (2009) Safety, tolerability, and pharmacokinetics of PA-824 in healthy subjects. *Antimicrob. Agents Chemother.*, **53**(9), 3720–3725.
[77] Garcia-Contreras, L., Fiegel, J., Telko, M.J. *et al*. (2007) Inhaled large porous particles of capreomycin for treatment of tuberculosis in a guinea pig model. *Antimicrob. Agents Chemother.*, **51**(8), 2830–2836.
[78] Dharmadhikari, A.S., Kabadi, M., Gerety, B. *et al*. (2013) Phase I, single-dose, dose-escalating study of inhaled dry powder capreomycin: a new approach to therapy of drug-resistant tuberculosis. *Antimicrob. Agents Chemother.*, **57**(6), 2613–2619.
[79] Tsapis, N., Bennett, D., O'Driscoll, K. *et al*. (2003) Direct lung delivery of para-aminosalicylic acid by aerosol particles. *Tuberculosis (Edinburgh)*, **83**(6), 379–385.
[80] Peloquin, C.A. (1991) Antituberculosis drugs: Pharmacokinetics. In: Heifets, L.B. (ed.) *Drug Suceptibility in Chemotherapy of Mycobaterial Infections*. CRC Press, Boca Raton, Florida, pp. 59–88.
[81] Davies, G.R. and Nuermberger, E.L. (2008) Pharmacokinetics and pharmacodynamics in the development of anti-tuberculosis drugs. *Tuberculosis (Edinburgh)*, **88**(Suppl. 1), S65–S74.
[82] Garcia-Contreras, L., Elbert, K., Hawi, A. *et al*. (2006) Pharmacokinetics of aerosolized Rifampicin large porous particles in the guinea pig. In: Dalby, R.N. *et al*. (eds) *Respiratory Drug Delivery*. Davis Healthcare International Publishing, LCC, River Grove, IL.
[83] Lee, S.H., Shin, J., Choi, J.M. *et al*. (2003) The impurities of capreomycin make a difference in the safety and pharmacokinetic profiles. *Int. J. Antimicrob. Agents*, **22**(1), 81–83.
[84] Garcia-Contreras, L., Muttil, P., Fallon, J.K. *et al*. (2012) Pharmacokinetics of sequential doses of capreomycin powder for inhalation in guinea pigs. *Antimicrob. Agents Chemother.*, **56**(5), 2612–2618.

8

Drug Particle Manufacture – Supercritical Fluid, High-Pressure Homogenization

Kimiko Makino[1] *and Hiroshi Terada*[2]

[1] *Faculty of Pharmaceutical Sciences, Tokyo University of Science*
[2] *Niigata University of Pharmacy and Applied Sciences*

8.1 Introduction

Current treatment for TB consists of frequent high dosing of combinations of oral anti-tuberculosis drugs. This method of treatment, however, causes systemic toxicity and undesired side effects while the intensive regimen leads to patient non-compliance [1]. To avoid systemic toxicity and maximize drug effectiveness, anti-tuberculosis drugs are also being formulated for direct delivery to the affected site, the deep regions of the lungs.

Rifampicin (RFP) as an anti-TB drug. RFP, one of the first-line medicines, is known to cause severe liver damage when combined with the use of isoniazid (INH) [2, 3]. In addition, RFP induces cytochrome P450, in particular CYP3A4 [4]. Therefore, the combined use of RFP and AIDS drugs which are metabolized mainly by CYP3A4 is contraindicated because the concentration of AIDS drug in the blood will decrease due to an RFP-mediated increase in CYP3A4 activity [5]. Furthermore, complications from AIDS have posed a problem in recent years. The risk of comorbid tuberculosis is very high for patients with AIDS whose immunity is compromised [2]. Nevertheless, RFP has a powerful anti-tubercular action, and therefore it is very important to reduce RFP's side effects and avoid drug interactions. Another problem is attaining an effective concentration of RFP in the lungs.

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

Usually, 450 mg of RFP is administered orally once a day. While the RFP concentration in liver reaches more than 30 mg/mL, the concentration of RFP in lung is only 1–5 mg/g, which is insufficient to provide antibacterial effects on *Mycobacterium tuberculosis* (MTB) located in alveolar macrophages (AMs) [6]. When RFP is orally administered, the major metabolite detected in urine is 25-deacetylrifampicin (DA-RFP) [7], with about 30% of unchanged RFP excreted in urine. The anti-tubercle bacillus activity of DA-RFP is reported to be lower than that of RFP [8–10]. Thus, it is important to develop an administration method to efficiently deliver unmetabolized RFP to the lungs.

Recently, microparticle and nanoparticle systems have been incorporated into inhalation delivery [11, 12]. Despite the ability of micro- and nano-particles to penetrate deep within the lung and their rapid uptake by alveolar macrophages, their delivery remains challenging. Particle agglomeration prohibits deep lung penetration, while unaggregated nanoparticles are exhaled due to their low inertia [6, 13, 14]. Therefore, micro- and nano-particle inhalation therapies often combine the active pharmaceutical ingredients (APIs) with pharmaceutically acceptable excipients or carriers. The blending of micro- and nano-particle APIs with pharmaceutical excipients for use in dry-powder inhalers (DPIs) can assist with processing properties such as flowability, promote dosing constancy, and provide a taste or sensation to assure the patient a dose has been received.

8.2 Preparation of Nano- and Micro-particles

8.2.1 Microparticles Prepared by a Supercritical Antisolvent–Drug Excipient Mixing (SAS–DEM) Technique

Ram B. Gupta's group has reported the preparation of rifampicin/lactose microparticle composites by a supercritical antisolvent–drug excipient mixing technique (SAS–DEM) technique for inhalation delivery [15]. RFP/lactose microparticle composites were prepared by dissolving RFP in a liquid solvent and spraying the solution into a high-pressure vessel containing supercritical CO_2 and suspended lactose particles, as shown in Figure 8.1. As the CO_2 extracts the liquid solvent, RFP microparticles precipitate to form a microparticle composite mixture with lactose particles. Homogeneous RFP/lactose composites consisting of spherical particles less than 8 μm in diameter were produced. No chemical interactions between RFP and lactose were observed, indicating that the therapeutic effectiveness of RFP was unaffected.

8.2.2 Nanoparticles Prepared by a Supercritical Fluid (SCF) Technique

Nanoparticles have been synthesized using a supercritical fluid (SCF) technique [16, 17]. Supercritical CO_2 and supercritical H_2O are extensively being used in the preparation of nanomaterials. The greatest requirement in the application of nanoparticles is its size- and morphology-control. RFP/poly (D,L-lactide) nanoparticles prepared by a supercritical assisted atomization (SAA) technique have been reported. Reverchon's group have shown that poly (D,L-lactide) and RFP were coprecipitated, and that nanoparticles with an average diameter ranging from 120 to 150 nm were obtained [17]. The encapsulated RFP was in an amorphous state. Also, ultrafine RFP particles have been prepared using a room temperature ionic liquid (RTIL) (1-ethyl-3-methylimidazolium methyl hydrogen phosphate) as

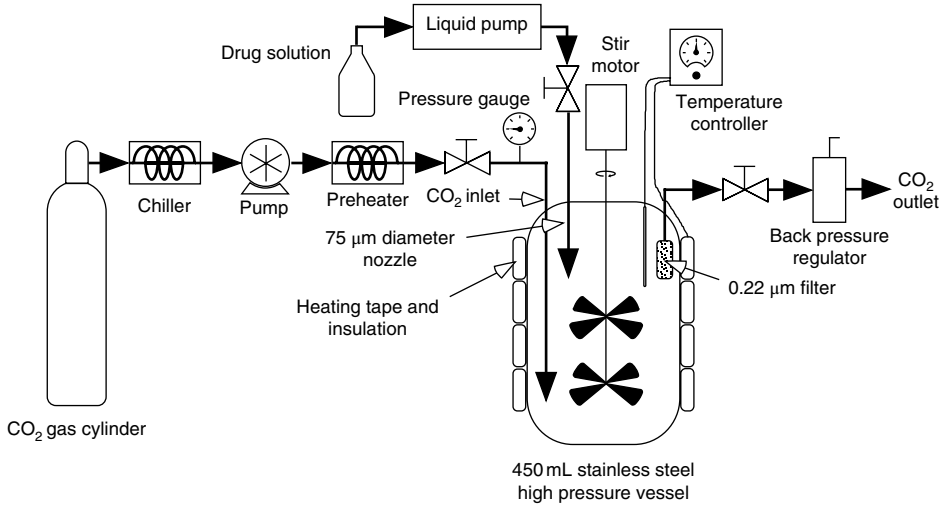


Figure 8.1 *Schematic of apparatus for SAS precipitation and simultaneous excipient mixing (SAS–DEM)*

a solvent and a phosphate buffer as an antisolvent. The solubility of RFP in this particular RTIL is higher than 90 mg/g at 30 °C, and the solubility is lower than 1 mg/g in water at 25 °C. Following introduction of an RFP solution in this RTIL into aqueous solution, the antisolvent can produce nanoparticles (260–360 nm) with or without hydroxypropyl methylcellulose as a stabilizer [18].

8.2.3 Nanosuspension

Nanonization reduces the mean size of solid drug particles to the nano-scale generally by top milling or grinding. This helpful methodology improves the solubility of drugs [19].

Clofazimine is a fat-soluble iminophenazine dye used in combination with rifampicin and dapsone as multidrug therapy (MDT) for the treatment of leprosy. It has been used investigationally in combination with other antimycobacterial drugs to treat *Mycobacterium avium* infections in AIDS patients and *Mycobacterium avium paratuberculosis* infection in Crohn's disease patients. Clofazimine nanosuspensions were produced by high-pressure homogenization and the formulation was optimized for lyophilization to treat *Mycobacterium avium* infection [20]. The particle size was almost 385 nm. The authors characterized their product by photon correlation spectroscopy, laser diffraction and coulter counter, and showed that the clofazimine nanosuspensions were suitable for intravenous (iv) injection with a particle size permitting passive targeting to the reticuloendothelial system. Following iv administration to mice of either the nanocrystalline or a control liposomal formulation at a dose of 20 mg clofazimine/kg bodyweight, drug concentrations in livers, spleens and lungs reached comparably high concentrations, well in excess of the minimum inhibitory concentration (MIC) for most *Mycobacterium avium* strains. When C57BL/6 mice were experimentally infected with *M*. avium strain TMC 724, nanocrystalline clofazimine was as effective as liposomal clofazimine in reducing bacterial loads in the liver, spleen and lungs of infected mice. Nanocrystalline suspensions of poorly

soluble drugs such as riminophenazines are easy to prepare and to lyophilize for extended storage and they represent a promising new drug formulation for intravenous therapy of mycobacterial infections.

8.2.4 Liposomes

Liposomes are vesicles in a nano- to micro-metre diameter range and are composed of a phospholipid bilayer surrounding an aqueous core encapsulating the desired drug. For prolonged sustainability and circulation time, liposome surfaces are PEGylated. Lung-specific stealth liposomes have been reported [21]. Modification of the surface of stealth liposomes by tagging *O*-stearylamylopectin (O-SAP) resulted in an increased affinity of these liposomes towards lung tissue of mice. Liposomes containing egg phosphatidylcholine (ePC), cholesterol (CH), dicetyl hydrogen phosphate (DCP), O-SAP and monosialogangliosides (GM1)/distearylphosphatidylethanolamine–poly(ethylene glycol) 2000 (DSPE–PEG 2000) were found to be most stable in serum. Tissue distribution of these liposomes showed more accumulation in lungs than in reticuloendothelial systems (RESs) of normal and tuberculous mice. Pre-administration of phosphatidylcholine (PC) and cholesterol (CH) (2:1.5) liposomes before the injection of lung-specific stealth liposomes further enhanced their uptake in lungs. *In vivo* stability of these liposomes demonstrated the slow and controlled release of their encapsulated contents. Isoniazid and rifampicin encapsulated in liposomes were less toxic to peritoneal macrophages as compared with free drugs. The liposomal drugs have also been shown to considerably decrease the bacterial load when compared with the free drug, improving the antimycobacterial efficacy and decreasing the toxicity of the encapsulated drug.

References

[1] Schreiber, J., Zissel, G., Greinert, U. *et al*. (1999) Lymphocyte transformation test for the evaluation of adverse effects of antituberculous drugs. *Eur. J. Med. Res.*, **4**, 67–71.
[2] Kameda, K., Kawabata, S. and Shirai, S. (1980) A case of acute hepatitis occurred during treatment with isoniazid and rifampicin. *Kekkaku*, **55**, 247–251 [see https://www.jstage.jst.go.jp/article/kekkaku1923/55/5/55_5_247/_pdf (accessed 9th May 2016). Summary and most references are in English; main text is in Japanese].
[3] Schaberg, T. (1995) The dark side of antituberculosis therapy: adverse effects involving liver function. *Eur. Respir. J.*, **8**, 1247–1249.
[4] Bouke, D.M.I., de Jong, C., Corbett, E.L. and Small, P.M. (2004) Clinical management of tuberculosis in the context of HIV infection. *Annu. Rev. Med.*, **55**, 283–301.
[5] Furesz, S., Scotti, R., Pallanza, R. and Mapelli, E. (1967) Rifampicin: a new rifamycin. 3. Absorption, distribution, and elimination in man. *Arzneimittelforschung*, **17**, 534–537.
[6] Heyder, J., Gebhart, J., Rudolf, G. *et al*. (1986) Deposition of particles in the human respiratory tract in the size range 0.005–15 µm. *J. Aerosol Sci.*, **17**, 811–825.
[7] Sano, M. and Hakusui, H. (1970) Urinary metabolites of rifampicin. *Jpn. J. Antibiot.*, **23**, 416–420.
[8] Maggi, N., Arioli, V. and Sensi, P. (1965) Rifamycins. XLI. 1 A New Class of Active Semisynthetic Rifamycins. N-Substituted Aminomethyl Derivatives of Rifamycin SV2. *J. Med. Chem.*, **8**, 790–793.
[9] Acocella, G. (1978) Clinical pharmacokinetics of rifampicin. *Clin. Pharmacokinet.*, **3**, 108–127.
[10] Swaminathan, S. and Nagendran, G. (2008) HIV and tuberculosis in India. *J. Biosci.*, **33**, 527–537.
[11] Swali, H., Semete, S., Kalombo, L. *et al*. (2009) *Nanomedicine for respiratory diseases*. Wiley Interdisciplinary Reviews. Nanomedicine and Nanobiotechnology, vol. **1**, pp. 255–263.

[12] O'Hara, P. and Hickey, A.J. (2000) Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: manufacture and characterization. *Pharm. Res.*, **17**, 955–961.
[13] Heyder, J., Gebhart, J., Rudolf, G. *et al*. (1984) Mathematical models of particle deposition in the human respiratory tract. *J. Aerosol Sci.*, **15**, 697–707.
[14] Prime, D., Atkins, P.J., Slater, A. and Sumby, B. (1997) Review of dry powder inhalers. *Adv. Drug Deliver. Rev.*, **26**, 51–58.
[15] Ober, C.A., Kalombo, L., Swai, H. and Gupta, R.B. (2013) Preparation of rifampicin/lactose microparticle composites by a supercritical antisolvent-drug excipient mixing technique for inhalation delivery, *Powder Technol.*, **236**, 132–138.
[16] Byrappa, K., Ohara, S. and Adschiri, T. (2008) Nanoparticles synthesis using supercritical fluid technology – towards biomedical applications. *Adv. Drug Deliver. Rev.*, **60**, 299–327.
[17] Labuschagne, P.W., Adami, R., Liparoti, S. *et al*. (2014) Preparation of rifampicin/poly (d, l-lactice) nanoparticles for sustained release by supercritical assisted atomization technique, *J. Supercrit. Fluid.*, **95**, 106–117.
[18] Viçosa, A., Letourneau, J.-J., Espitalier, F. and Inês Ré, M. (2012) An innovative antisolvent precipitation process as a promising technique to prepare ultrafine rifampicin particles. *J. Cryst. Growth*, **342**, 80–87.
[19] Rabinow, B.E. (2004) Nanosuspensions in drug delivery. *Nat. Rev. Drug Discov.*, **3**, 785–796.
[20] Peters, K., Leitzke, S., Diederichs, J.E. *et al*. (2000) Preparation of a clofazimine nanosuspension for intravenous use and evaluation of its therapeutic efficacy in murine Mycobacterium avium infection. *J. Antimicrob. Chemother.*, **45**, 77–83.
[21] Deol, P. and Khuller, G.K. (1997) Lung specific stealth liposomes: stability, biodistribution and toxicity of liposomal antitubercular drugs in mice. *Biochim. Biophys. Acta*, **1334**, 161–172.

9

Spray Drying Strategies to Stop Tuberculosis

Jennifer Wong[1], *Maurizio Ricci*[2] *and Hak-Kim Chan*[1]

[1] *Advanced Drug Delivery Group, Faculty of Pharmacy, University of Sydney, Australia*
[2] *Dipartimento di Scienze Farmaceutiche, Università degli Studi di Perugia, Perugia, Italy*

9.1 Introduction

The current recommended treatment for tuberculosis (TB) involves a six-month regimen of two to four antibiotics (isoniazid, pyrazinamide, rifampicin, and/or ethambutol). Hence, the major barriers to patient adherence are the lengthy treatment times and co-administration of multiple high-dose tablets that leads to unwanted side effects. The need to simplify and shorten TB treatment has long been a priority [1, 2]. While this could be achieved by developing newer and better agents, the same objective could be met by enhancing the bactericidal activity of therapeutics already in use or in development [3, 4]. Consequently, there has been renewed interest in developing TB therapeutics that directly target the site of infection with the aim to increase local therapeutic effect and reduce overall systemic exposure [1, 4–12].

Spray drying technology is well placed to meet the challenge of developing targeted drug delivery systems for TB. First, it is a unique processing method that allows control over critical fine-particle design features [13]. Secondly, it is an established technique that continues to be attractive for its relative simplicity, cost-effectiveness, and scalability for commercial production [14–17]. The expansive list of patents recently registered with the US Patent Office exemplifies that there is continuous worldwide interest in spray drying technologies for various pharmaceutical applications [18]. With regards to the development of

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

therapeutics for TB treatment and prevention, spray drying has been largely used to repurpose therapeutics as superior inhalation products with improved safety, efficacy, and convenience for patients [19]. Despite the fact that TB disseminates via the respiratory tract and primarily affects the lungs, there is not one anti-TB aerosol product that is commercially available. This chapter will give an overview of the spray drying process (including details about the hardware, process parameters, and key theories), highlight the recent technological advances that could facilitate the development of innovative therapeutics for TB, and evaluate the anti-TB formulations produced by spray drying.

9.2 Overview of Spray Drying

Spray drying is a mild, ultra-fast, convective drying process aimed to produce engineered dry powders, agglomerates or granules, starting from a solution, suspension or semisolid, rapidly dried under a hot gas stream. This is probably one of the most common and helpful method used to dry temperature-sensitive materials like foods, chemicals, and pharmaceuticals. Historically, this technique has its roots in the US patent no. 125,406 issued in 1872 entitled "Improvement in Drying and Concentrating Liquid Substances by Atomizing" [20]. Thus, Samuel Percy can be considered the inventor of spray drying technology, declaring in the patent: "Be it known that I, SAMUEL R. PERCY, of the city, county, and State of New York, have invented a new and Improved Process for the Simultaneous Atomizing and Desiccating of Fluid and Solid Substances, and its application to the purpose of the exhaustion of moisture from such substances".

Because of practical reasons at the time, such as low process efficiency, continuous process performance, as well as safety problems, there was very limited commercial utilization of this technology for a long time. Only during the second decade of the twentieth century did a consistent degree of evolution in spray dryer design enable the large-scale application of the process. Specifically, it was mainly focused in the dairy industry and dried milk was the first commercial product obtained by this technology [21, 22]. However, the great input to this technology came in the 1940s when Spraying Systems Co. developed the first line of commercially available nozzles for spray drying. Interestingly the term "SprayDry®" was first used by Spraying Systems Co. in 1943 and became a registered trademark of the company in 1951. Since spray drying can be used to preserve food in the dry state and also provides the advantage of reducing weight and volume, this process blossomed during World War II when there was a considerable need to transport large amounts of food such as milk, eggs, and other materials rapidly and in small volumes. Since then, the technology evolved rapidly and intensive research allowed the spray drying technique to meet a wide variety of applications, including the production of instant coffee [23], fertilizers [24], encapsulated food ingredients [25–29], and pharmaceuticals [18].

In the pharmaceutical field, spray drying is commonly used to produce powders forming the basis of dry dosage forms for pulmonary [30, 31] and nasal [32–36] applications. In this respect, particles must meet certain features in flowability, dispersibility, and (in the case of respiratory delivery) suitable aerodynamic properties such as particle size lower than 5 μm and low particle density for easy transport to the lung [4, 37–42]. Microencapsulation of active pharmaceutical ingredients (APIs) [43–47], as well as actives for cosmetic applications [48], in biodegradable polymers for prolonged release and improved bioavailability

represent another interesting application of this technology. Besides, spray drying is a very suitable alternative to the freeze drying technique in manufacturing heat-sensitive vaccines as powders [49–51]. Owing to the rapid drying kinetics, a spray drying process, ideally, can be considered the technology of choice in producing amorphous pharmaceutical solids to improve the bioavailability of poorly soluble APIs [52–55]. The next section will give an overview of the spray drying process, including details about the hardware, process parameters, and key theories.

9.2.1 Advantages of Spray Drying

Spray drying technology offers several advantages over the other drying processes:

- It covers a wide range of applications from pharmaceutical to ceramic powder production.
- The equipment can be designed to meet a wide range of production needs (from lab to industrial scale).
- Feed rates can range from a few pounds to a hundred tons per hour.
- Feedstock can be in solution, slurry, paste, gel, suspension, or melt form.
- It is possible to maintain constant final product specifications when the process conditions are held constant.
- Different product types: granules, agglomerates, powders, and composite materials can be produced.
- Ease of operation that is adaptable to full automatic control.
- Suitable for both heat-resistant and heat-sensitive products.
- No contact exists between material and metal surfaces until dried, reducing potential corrosion problems.
- It can satisfy aseptic/hygienic drying conditions.

9.2.2 Hardware

Although many spray dryer designs are available on the market meeting different product specifications, a typical drying process basically consists of three stages (Figure 9.1):

a. Atomization.
b. Drying.
c. Particle collection.

Each of the stages above are, along with the physical and chemical properties of the feed, critical for the final product properties and, for this reason, for each of them a wide variety of process designs are available depending on specific needs or applications.

9.2.2.1 Atomization

The atomization stage transforms the initial liquid feed (solution, suspension, dispersion, or emulsion), generally concentrated prior to its introduction into the spray dryer, in small droplets of desired size by appropriate atomizers. The aim of this stage is to create a maximum heat-transferring surface of contact between the drying gas and the liquid feed, providing the right conditions to an ultra-fast (few seconds) evaporation of the solvent [56]. As a consequence, the drying material does not reach the inlet temperature of drying gas, thus avoiding any thermal degradation process of heat-sensitive substances.

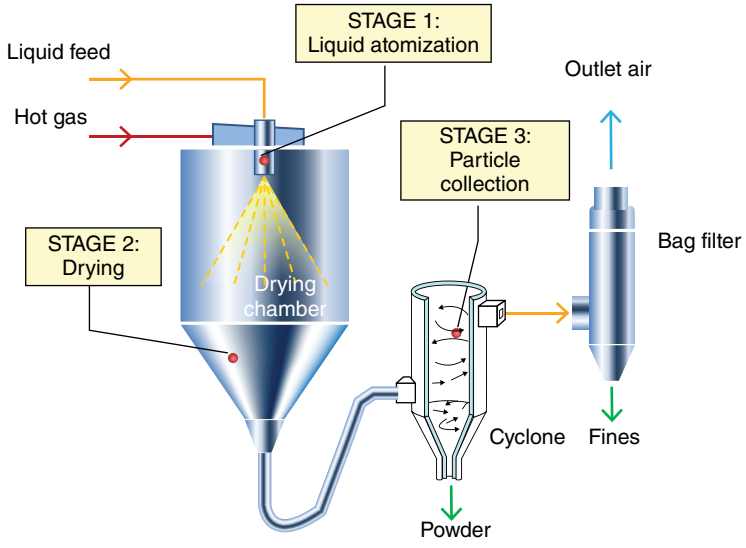


Figure 9.1 *Schematic of a spray dryer unit. Diagram not drawn to scale*

Table 9.1 *Median droplet size obtained from different atomization devices [59]*

Atomization device	Median droplet size (μm)
Rotary atomizer (wheel)	10–200
Pressure nozzle	30–350
Pneumatic nozzle (two/three-fluid)	5–100

Atomization is the critical stage of the entire process in fact, the conditions of the feed atomization define the size distribution of the formed droplets and therefore the physical properties of the final dry particles. Achieving the desired level of atomization requires maintaining a balance of the fluid viscosity and fluid flow rate on one side with atomization energy on the other side, a change in any parameter affecting the atomization. Balancing the equilibrium with an opposing change can return the atomization to the desired level.

The atomizers can be classified according to the type of energy utilized and include [17] rotary atomizers (centrifugal energy), hydraulic or pressure nozzles (pressure energy), two-fluid or pneumatic nozzles (kinetic energy), and more recently four-fluid nozzles with in-line mixing have been developed to produce composite particles [57, 58]. There are several configurations for each type of atomizer, producing different sprays with specific features.

Each kind of atomizer has to fulfil several important functions such as: to form droplets that must be neither too large that they could not be completely dried out, nor so small as to make difficult their recovery; to distribute the formed droplets evenly in the drying chamber so that they can come thoroughly into contact with the hot gas; finally, to control the rate at which the feed material is sprayed into the dryer. However, for each feed stream property the droplet size depends on the type of nozzle (Table 9.1).

9.2.2.1.1 Rotary Atomizer In this device, the feed to be atomized is introduced at the center of a wheel rotating at high speed. The centrifugal force generated pushes the liquid toward and beyond the disc perimeter, thus causing the formation of a cloud of droplets. Droplet sizes are controlled by varying the rotating speed of the wheel, feed rate, feed physical properties, and atomizer design. The degree of atomization, in turn, affects the properties of the final dried product. It was found that maximum particle size was achieved by reducing the atomizer speed [60].

The mean size of the droplets is inversely proportional to the wheel speed and directly proportional to the feed rate and its viscosity. Other factors influencing droplet size are solid content and surface tension. For a better control over the dispersion properties, most disc atomizers present grooves whose shapes depend on the dry particle parameters desired [61] and the properties of the feedstock.

Rotary atomizers are reliable, easy to operate, and can handle high feed rates and abrasive feeds. Moreover, they offer the most effective means of atomization in spray drying technology. However, in some cases, product can get stuck on the walls of the drying chambers can be formed, and this can be a serious problem for expensive pharmaceutical products.

9.2.2.1.2 Hydraulic or Pressure Nozzles In this kind of nozzle (one-fluid nozzle), the spray is created by forcing the feed at high pressure (20–100 bar) through a tiny orifice of 0.4–4 mm diameter. The principle of pressure atomization is based on the conversion of the high-pressure energy supplied by a pump into kinetic energy (velocity). In fact, by Venturi effect, when the fluid at high pressure passes through the orifice a pressure drop is observed and the corresponding increase of velocity breaks up the formed thin liquid film into droplets. Mean size of droplets is related to liquid properties such as viscosity, surface tension, density, and capacity (quantity per unit of time). Specifically, it is proportional to the feed rate and viscosity and inversely proportional to nozzle pressure. Therefore, in order to reduce the droplet size for a given capacity a smaller orifice and a higher pump pressure must be utilized to achieve the same mass flow rate through the nozzle. Masters [59] introduced the following correlation [Equation (9.1)] to predict the mean droplet diameter produced by pressure nozzle.

$$d_{32} = \frac{2774 \cdot Q^{0.25} \cdot \mu}{\Delta P^{0.5}} \tag{9.1}$$

where:

d_{32} = Sauter mean diameter
Q = volumetric feed rate (ml/s)
μ = feed viscosity (MPa.s)
ΔP = operating pressure (kPa).

Pressure nozzles used in spray drying are also known as "vortex" nozzles because most of them contain a swirl chamber giving the liquid a rotation, so that it will leave the orifice as a hollow cone. They can be used to atomize all types of feeds, although when suspensions are sprayed the suspended material should be controlled to avoid nozzle clogging and erosion as well, and/or pump failure. These nozzles are also well suited to atomize liquids with viscosities up to several hundred centipoises and they are successfully used in

counter-current spray dryers, and in systems using multiple nozzle lances. Sprays from pressure nozzles are generally less homogeneous and coarser than are sprays from rotary atomizers, thus producing coarser, free-flowing powders.

9.2.2.1.3 Pneumatic Nozzles (Two-Fluid) These operate using compressed air to create high frictional forces over liquid surfaces, thus determining feed reduction into spray droplets. In this system neither the liquid nor the air requires very high pressure, usually ranging between 200 kPa and 350 kPa. Inside the nozzle there are two ducts: the central one, through which passes the feed to be atomized, is surrounded by another conduit loop in which air is introduced under pressure. The air outlet of the nozzle forms a cone at the apex of which the air velocity is maximum and the pressure is minimum so that it sucks the liquid coming out at the center of the nozzle. The interaction of the liquid with the air causes the atomization of the liquid into small droplets. Particle size is controlled by varying the ratio of the compressed air flow to that of the liquid. The mean spray size produced by pneumatic nozzle atomization follows the relationship given in Equation (9.2) [59]:

$$d_{32} = \frac{\mathrm{A}}{\left(u_{\mathrm{rel}}^2 \cdot \rho_a\right)^{\alpha}} + \mathrm{B}\left(\frac{M_{\mathrm{gas}}}{M_{\mathrm{liq}}}\right)^{-\beta} \tag{9.2}$$

where:

α and β are functions of nozzle design
A and B = constants involving nozzle design and liquid properties
u_{rel} = the relative velocity between gas and liquid (m/s)
ρ_{a} = density of air
M_{gas} and M_{liq} = mass flow rate of compressed air and feed, respectively.

This type of atomizer is particularly useful for materials of high viscosity, which produce particles of medium coarseness. In addition, since the liquid leaves the atomizer at a relatively low velocity and therefore has a shorter flight path, it requires a smaller drying chamber, which makes it ideal to be used in pilot- or laboratory-scale drying processes. Pneumatic nozzle atomization has the disadvantage of being expensive to operate, especially for medium or large production scales. In fact, owing to the high operating cost associated with providing a high-velocity air stream, the energy required is generally two to three times that for pressure nozzles [62]. In the pharmaceutical industry, the most commonly used nozzles are pressure nozzles and pneumatic nozzles, due to their scalability and reduced tendency to form deposits on the walls in comparison with rotary nozzles.

9.2.2.2 Drying

The central element of a spray dryer is the drying chamber where atomized liquid comes in contact with hot gas (air or nitrogen), resulting in the evaporation of more than 95% of the water contained in the droplets in a few seconds. There are different modes of contact which influence the flow behavior of the droplet during the drying process, the evaporation rates and product temperatures in the dryer, affecting directly the end-dried product properties. The three possible patterns are: co-current, counter-current, or mixed (a combination of the two patterns).

9.2.2.2.1 Co-current Flow Dryer In this kind of dryer, the feed is sprayed through the drying chamber in the same direction as the flow of the heated drying gas. Co-current dryers are ideal for thermolabile materials such as enzymes, peptides, and proteins. In fact the hottest drying gas, coming into contact with the droplets, determines a rapid evaporation of the water that will mix with the air stream. During this process the gas moisture content will increase, while its temperature will decrease since part of the latent heat of water vaporization comes from the air. Therefore, the product does not suffer from heat degradation because the droplet temperature is low during most of the evaporation time. Once the product moisture content reaches the target level, the temperature of the particle does not increase greatly because the surrounding air is now much cooler. When operating with a rotary atomizer, the air disperser gives rise to a high degree of air rotation, thus making the temperature uniform throughout the drying chamber. The particle size of the final powder can be controlled by changing wheel speed.

9.2.2.2.2 Counter-current Flow Dryer In this dryer system, the atomized feed and heated drying medium move in opposite directions through the drying chamber with the atomizer generally positioned at the top while the drying air enters from the bottom. The exposure of the sprayed feed to the highest air temperatures occurs when the material is almost dry and therefore this drying system is suitable for thermally stable substances (soaps and detergents, for example) [63]. Counter-current dryers normally use nozzles to atomize the feed because the energy of the spray can be directed against the air movement.

9.2.2.2.3 Mixed Flow Dryer This type combines both co-current and counter-current flow with several variants including the so- called "fountain spray drying" that provides the gas entering from the top and the atomized liquid from the bottom. Similarly to the counter-current design, the partially dried particles enter the hottest region of the drying chamber near the drying medium's dispenser and for this reason the mixed flow dryer is not suited for heat-sensitive products.

9.2.2.3 Particle Collection

Finally, dry particles must be collected from the air stream in efficient manner for both economic and environmental reasons. They can separate out from the drying gas directly within the drying chamber where most of the particles are discharged from its base through a suitable airlock, such as a rotary valve, while the cooled gas stream, containing the smallest particles, is conveyed through a side outlet in other separators. Alternatively all the particles are conveyed to and separated out in a cyclone or bag filter.

The cyclone separator uses centrifugal acceleration to replace gravitational acceleration as separating force. In fact, particle-laden gas enters at high velocity (approximately $30.5\,m\,s^{-1}$) tangentially through a vertical cylinder with a cone-shaped bottom, at one or more points flowing in a helical pattern (like a sort of "mini tornado") beginning at the top and ending at the bottom of the cyclone. In this rotating stream, larger particles under the centrifugal force applied do not follow the tight curve of the stream, and thus, colliding with the chamber wall, they lose speed and fall to the bottom of the cyclone by gravity. Lighter particles continue on the helical air flow path along the walls of the cyclone and, as soon as the flow reaches the conical end of the cyclone, the rotational radius of the stream is reduced, thus separating smaller particles.

However, a portion of the lighter particles will be dragged outwards by the gas reversing up through the inner chamber and out from the top of it. Cyclone separators' efficiency is determined by the cyclone geometrical parameters, the density of the solids, and the rotational velocity of the air stream and, in order to improve it, several cyclones can be placed in series. Unless very small units are used, their efficiency is low in collecting powders smaller than 5 μm, which are of great interest in pulmonary delivery, capable of reaching the deep part of a patient's lungs [64]. Besides, it has been observed that a low product yield (normally only 30–40%) is associated with bench-top spray dryers for protein powder preparation [65, 66]. Wet scrubbers are often used for a secondary air-cleaning system in conjunction with the cyclone to purify and cool the air. They work by dissolving any dust powder left in the air stream into either water or the feed stream sprayed through the air. In this way they recover not only most of what would be lost product, but also approximately 90% of the drying energy normally lost in exiting air.

9.2.3 Spray Dryer Classifications

It is possible to classify spray dryers depending on the type of cycle, type of stage, and type of position (Figure 9.2).

9.2.3.1 Classification Based on the Type of Cycle

9.2.3.1.1 Open Cycle This is the standard design and is the most used: the air is drawn from atmosphere, is heated, mixed in the drying chamber with the feed, and, after exhausting, cleaned by means of cyclones, and finally is discharged to atmosphere [67]. Direct and indirect heating are applicable. Since the heated air is exhausted without recycling, this type of layout is an energy-wasting system. The use of organic solvents and drying gases other than air is normally not recommendable because of economical as well as ecological problems. Therefore, open-cycle systems are applied to spray dry aqueous feeds.

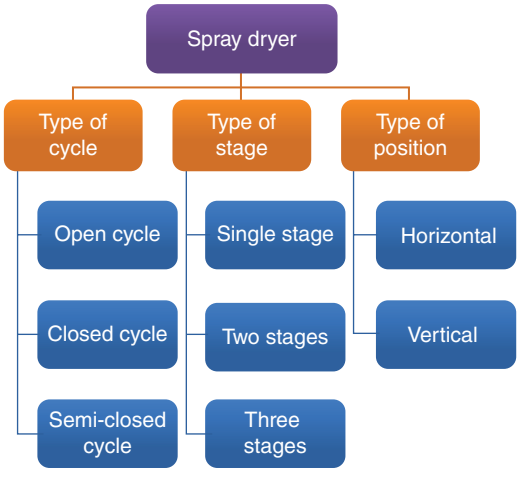


Figure 9.2 *Spray dryer classifications*

9.2.3.1.2 Closed Cycle This type recycles the drying gas, commonly an inert gas such as nitrogen, and it is the dryer of choice when:

- Flammable organic solvents are used.
- Complete recovery of solvent is required.
- Processing highly oxidizable product that should not come in contact with oxygen during drying [68].
- The products are toxic.
- Pollution caused by the vapor, particulate emissions, or odor must be avoided; in this respect, being that dried products are the only output, this layout can be considered environmentally friendly.
- Explosion risks must be eliminated.
- Besides, this layout is well suited to operate under aseptic conditions [69].

9.2.3.1.3 Semi-Closed Cycle This type is in between open and closed cycle designs and it is not gas tight. This design was developed to improve thermal efficiency by utilizing heat in the exhaust gas leaving the dry collectors. The drying medium can be partially (recycling of up to 60% of the exhaust air as inlet air) or mostly recycled and its heating, before re-entering the drying chamber, can be direct, by using oil or gas, or indirect. Direct heating should be avoided in drying food products because of the possible contamination by the nitrogen-containing compounds that can be formed during the process.

9.2.3.2 Classification Based on the Type of Stage

9.2.3.2.1 Single Stage The single-stage dryer is the most used design. In this dryer, the moisture reduction to the target (2–5% by weight) is obtained in one pass through the dryer system. The rate of evaporation is particularly high only at the beginning of the process, while it gradually decreases because of the reducing moisture content of the particle surfaces. In order to complete the drying process in just one stage, a relatively high outlet temperature is required during the final drying phase, resulting in possible heat damage to the dry particles that are forming.

9.2.3.2.2 Two Stages Although the single-stage spray dryer may produce dried products with the desired characteristics, in order to improve product quality and obtain higher thermal efficiency, and lower the costs, a two-stage drying system has been developed. It consists of a spray dryer with an external fluid bed placed below the drying chamber. The product can be removed from the drying chamber with a moisture content higher than the final product (typically 5–10%). In a second stage the moisture content is further reduced in the external fluid bed (fluidized bed or a vibrating bed dryer) where the residence time of the product is longer and the temperature of the drying air lower than in the spray dryer. This two-stage drying results in a better heat economy and it is suitable for many products which are heat sensitive.

9.2.3.2.3 Three Stages The above principle puts the basis for the development of the three-stage dryer. In this configuration, the second stage is a fluid bed built into the cone of the spray drying chamber. As a consequence, it is possible to achieve an even higher moisture content in the first drying stage and a lower outlet air temperature. This fluid bed is

known as an "integrated fluid bed". The inlet air temperature can be raised, resulting in a larger temperature difference and improved efficiency in the drying process. The third stage is again an external fluid bed, static or vibrating, for final drying and/or cooling of the powder. The three-stage dryer shows some advantages such as higher quality powders with much better rehydrating properties and lower energy consumption.

9.2.3.3 Classification Based on the Type of Position

9.2.3.3.1 Horizontal In this kind of spray dryer, the chamber has the form of a rectangular box with flat or a "V"-shaped bottom. Normally, the feed is sprayed horizontally, with the dried particles falling to the floor, from where they are removed by means of a conveyor. Box dryers are usually small and the particle- residence time relatively short, requiring the use of low flow nozzles, which produce relatively small particles.

9.2.3.3.2 Vertical The chamber of a vertical (tower) dryer has the classical form of a cylinder with a cone-shaped bottom. Spray nozzles may be located at the top (co-current flow) or bottom (counter-current or mixed flow) of the chamber. Inlets for the drying air may be located at the top, bottom, or side of the chamber. Vertical spray dryers are usually large and the residence time of sprayed particles is relatively long, allowing the use of higher-flow nozzles which produce relatively large particles.

9.2.4 Process Parameters

In order to obtain a product with the desired characteristics, a series of processing parameters must be considered and carefully checked, such as atomization pressure, drying temperature (inlet/outlet), drying gas air flow rate, feed flow rate, feed properties, as well as environmental conditions [70, 71].

9.2.4.1 Inlet/Outlet Temperature

The inlet temperature of the drying air is the temperature of the drying medium contacting the feed and is considered the most important parameter determining the internal structure of the resulting spray-dried particles. As inlet temperature increases, outlet temperature proportionally increases. The higher the inlet air temperature, the faster the rate of moisture evaporation. A higher temperature reduces the relative humidity in the drying gas and the powder becomes dryer, less sticky and increased yields are observed [55].

The outlet temperature is that of the air with the solid particles before entering the cyclone and being the resulting temperature of the heat and mass balance in the drying chamber it is, in practice, the highest temperature to which the product may be heated and it cannot be regulated. The outlet temperature is the result of the combination of the following parameters: i) inlet temperature, ii) aspirator flow rate, iii) peristaltic pump setting, and iv) concentration of the material being sprayed. An increase in inlet air flow rate or its temperature signifies an increase in the amount of energy available for evaporation, and since there is no change in the amount of water to be evaporated the energy used for the evaporation will be the same. As a result there is energy in excess which is seen as a rise in the outlet air temperature. The optimal choice for the temperature difference between the inlet and the outlet temperature is one of the most important points to consider when spray drying: increasing

the temperature difference while holding the inlet temperature constant increases the residual moisture content in the final product as well as the spray flow rate of the device.

9.2.4.2 Drying Gas Air Flow Rate

This is the amount (volume) of drying air supplied to the system per unit time. It determines the drying level of the product and its separation in the cyclone. The lower the drying gas air flow rate, the slower the movement of the product particles through the system and the longer the action of drying. Generally, it is suggested to adjust this parameter to the maximal value available in order to maximize the cyclone operation efficiency [72]. Higher amounts of drying air result in better atomization of the liquid stream (smaller droplets) and, as a consequence, smaller particles. The pressure under which the gas is supplied depends on the nozzle.

9.2.4.3 Feed Flow Rate

Feed flow rate governs the atomization pattern and droplet-size distribution, and the time period a particle remains in the drying chamber, cyclone and bag filters during the spray drying operation. An increase in feed flow results in a shorter contact time between the feed and drying air, which makes the heat transfer less efficient, thus causing lower water evaporation. As a consequence, more energy is needed to evaporate the droplet to leave particles, with a consequent decrease of outlet temperature. This also results in an increased absolute humidity and relative humidity as well, which cause the moisture content in the final powder to increase. Holding the liquid pressure constant, increasing the feed flow yields larger droplets because of the greater amount of liquid to be atomized.

9.2.4.4 Feed Solids Content

The change in feed solids content results in a change in outlet temperature due to the same reason as for the feed flow rate. Increasing solid concentration means less liquid to vaporize and increases the outlet temperature. When maintaining constant atomization and inlet temperature, an increase of solid content generally results in a higher particle density as well as in bigger particles that are easier to separate and so the yield increases. Generally, a high solid content can yield an excessive viscosity, resulting in poor atomization process that, in turn, will negatively affect the formation of droplets with spherical shape as well the uniformity of the final particles.

As the feed liquid viscosity increases, the energy supplied to the nozzle must overcome larger viscous forces and this reduces the energy available for breaking up the droplets, resulting in larger droplets. The liquid viscosity also affects the spray pattern: a more viscous liquid typically produces a narrower spray angle. However, the general rule is that the solid content should be as high as possible to minimize the evaporation energy required and hence the costs. For this reason, usually the feed is concentrated before spraying.

9.2.4.5 Ambient Humidity

The ambient humidity is viewed to have a minor impact on the temperature of the air in the chamber. Nevertheless, it affects the spray dryer's ability to dry a product. Excessive humidity results in moist particles, which could adhere to the glassware thereby decreasing the yield. This problem is particularly important for highly hygroscopic products: in such cases, dehumidification of the inlet air is necessary prior to use.

9.2.4.6 Ambient Temperature

The ambient temperature affects the moisture content of the inlet air. At constant relative humidity, a higher ambient temperature results in an increase of moisture content of the drying air. Besides, the higher ambient temperature affects positively the heat loss since heat transfer through the chamber wall is less significant when the difference between the wall temperature and ambient temperature is low.

9.2.5 Particle Formation Mechanism

It is well known that final particle properties such as size, density, porosity, surface roughness, and surface composition are related to the interplay between process parameters and the liquid-feedstock properties [73]. To design particles with better properties it should be important to have detailed information about the single-droplet drying process whose mechanism, although extensively studied and reviewed [17, 74], is not yet completely clear because of the complex, rapid, and simultaneous heat and mass transfer between feed and the heating gas. Besides, a theoretical description of such a processes is a challenging problem due to the many interacting physical mechanisms involved [75–78]. From a qualitative point of view, before becoming a powder, a liquid feedstock undergoes a series of complex events, during the entire spray drying process, affected by many interconnected variables, as reported by Hecht and Bayly [79].

The drying profile of the droplet generated by nebulization can be divided into several stages [73, 80–82] as reported below (Figure 9.3):

1. After atomization, a droplet experiences initial heating and evaporation occurs. The temperature increases until it is very close to the so-called wet bulb temperature of the solvent [83]. During this stage the evaporation is slow because most of the heat transferred to the droplet from the surroundings is used for droplet heating.
2. This stage is characterized by rapid solvent evaporation. Since most of the heat transferred to the droplet is used for the solvent evaporation, the temperature remains constant close to the wet bulb temperature. The droplet shrinks as the solvent evaporates from the

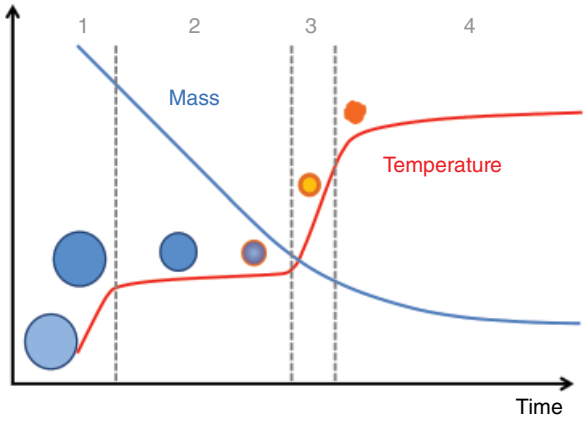


Figure 9.3 *Mass and temperature changes during single-droplet drying inside a spray dryer. (See insert for color representation of the figure.)*

surface and the solute within the droplet arranges according to its diffusion properties. Thus, the droplet temperature may increase slightly because of minor evaporation hindrance [84].

3. Drying precipitation occurs at the droplet surface and a solid phase forms around a wet core because the droplet diameter decreases faster than the solute can diffuse into the droplet center.
4. This is a falling-rate drying step [85] because the thickening crust represents a significant resistance to water transport from the center to the surface. The decrease in evaporation rate induces an increase in droplet temperature because less energy is needed for evaporation. During this step the crust formed thickens towards the droplet center until a particle has formed. As the solvent boundary proceeds inward, the forming particle surface may harden or collapse according to particle properties and the value of the solvent evaporation/solute diffusion ratio. Such a value is quantitatively represented by the Péclet number (*Pe*) [17, 81, 82]. $Pe > 1$ determines a 'buckling regime' and the formation of hollow or crumpled particles is usually observed, whereas $Pe < 1$ values correspond to a 'non-buckling regime' with production of more homogeneous spherical particles. *Pe*, together with the atomizing air/liquid feed ratio (ALR), determines droplet size and density and the final properties of spray-dried particles [86, 87]. The resulting particles may be relatively uniform hollow spheres, or porous and irregularly shaped.

One of the most sensitive aspects of the entire process is certainly controlling the feed liquid atomization process. In this respect, the selection of the most suitable atomizer is the fundamental step since:

- It controls liquid flow rate into the dryer.
- It breaks the liquid into droplets, forming a greater surface area.
- It disperses these droplets into a specified spray pattern.

This process therefore creates the conditions for proper droplet contact with the heated air and. consequently, an efficacious drying process and a final product with the required properties. When the droplet size is too big, an increase in moisture content in the dryer can easily be observed, which, in turn, can cause material build up in the dryer as well as high moisture content in the final product. Also, droplets which are too small should be avoided, since the fast moisture evaporation can cause changes in color and taste of the final product or can produce excessive dusty particles. Droplet size and droplet size uniformity will vary not only with the spray nozzle design but also with several operating parameters: liquid pressure, flow rate, and spray angle, as well as feed properties such as viscosity, surface tension, and density. Flow rate has a direct relation to droplet size: an increase in flow rate will increase the droplet size. Pressure has an inverse relationship effect on droplet size: an increase in pressure will reduce the droplet size. Spray angle has an inverse relationship effect on droplet size: an increase in spray angle will reduce the droplet size. Viscosity and surface tension increase the amount of energy required to atomize the spray: an increase in any of these properties will typically increase the droplet size. To conclude, as the operating parameters and liquid properties are strictly interrelated, it is important to change any spraying conditions with care. Besides, proper dryer maintenance will help in in guaranteeing droplet size and final product particle quality.

9.3 Advances in Spray Drying Technology

Even though spray drying technology is not new, it continues to be relevant and increasingly popular. In particular, the versatile process has become a powerful particle engineering tool with demonstrated unique control over particle size, particle morphology, and density [16, 17]. The past decade has seen the introduction of advances in spray drying technology: namely, the 'Quality by Design' approach, the Nano Spray Dryer B-90, and novel multi-channel nozzles.

9.3.1 The 'Quality by Design' Approach

Process development in spray drying has traditionally been empirical and experimentally driven. However, regulatory authorities such as the International Conference on Harmonization and the US Food and Drug Administration have called for the pharmaceutical industry to gain comprehensive understanding of their manufacturing processes, together with an accurate estimation of their robustness and reliability [88, 89]. The emphasis has been placed on the 'Quality by Design' (QbD) concept, where quality should be built into the product, not tested afterwards. In order to meet these new requirements, the development of commercially viable products necessitates robust design approaches to meet release specifications at minimum expense in terms of time, resources, and cost [90].

Design of experiments and various statistical analyses are methods that have been used to optimize process and formulation parameters [47, 88, 91–96]. While these techniques give insight into the relationship between process parameters and product attributes, a large number of experiments need to be performed to characterize a narrow design space that would have inevitable uncertainties and interactions [97]. These uncertainties are greater when little or nothing is known about a specific process. Hence, labor-intensive experiments cannot be avoided. Furthermore, these are not based on fundamental theory and have limited ability to translate across scales and formulations, especially when the influence of process parameters may not be linear [92].

Consequently, efforts have intensified to understand and control particle-formation processes to facilitate rational development and guarantee their quality [74, 98, 99]. Two widely accepted models deserve particular mention. First, the geometric diameter of a spray-dried particle (d) can be calculated using Equation (9.3):

$$d = \sqrt[3]{\frac{C}{\rho}} D \tag{9.3}$$

Here C is the drug solution concentration, D is the diameter of atomized droplets, and ρ is the density. This equation shows that changes in the concentration of the feedstock and the atomization process, which determines the diameter of the droplets (D), directly affects the particle size. Secondly, several authors emphasize the usefulness of *Pe* for the prediction of particle morphology during spray drying. *Pe* is defined as the ratio between droplet evaporation rate κ and diffusional motion of the solutes D_i, Equation (9.4) [73, 100]:

$$Pe_i = \frac{\kappa}{8D_i} \tag{9.4}$$

Small *Pe* less than 1 indicate that the diffusional motion of the solutes is fast compared with the velocity of the receding droplet surface during evaporation. In this case, solid particles with a density close to the true density of the dry components are likely to form. On the other hand, high *Pe* larger than 1 suggest the receding surface moves faster than the motion of the solutes. Depending on the nature of the formulation, different solidification mechanisms are triggered so the resulting particles can have a range of different morphologies. These may include solid hollow spheres, if the shell becomes rigid quickly and does not buckle or fold, as well as dimpled or wrinkled particles. Particle formation during spray drying has been described in detail by Vehring [17]. But more importantly, the establishment of such models clearly signals a shift in the design of fine particles by spray drying. That is, from being empirical and experimentally driven to having an engineering basis. This trend is expected to pick up as we gain better understanding of the inter-related process and formulation parameters [73, 101].

Additionally, fundamental engineering models, ranging from steady-state and equilibrium-based approaches to rate-based and computational fluid dynamics (CFD), have been applied to understand the spray drying process. In particular, CFD has been proposed as a useful scale-up tool since the 1990s [75]. It is a simulation method that combines powerful computing with applied mathematics to model fluid-flow situations and accounts for aspects such as heat and mass transfer, including basic drying kinetics, reaction engineering, particle engineering, and process control [102, 103]. However, the biggest danger is that the outcome of the simulation would be invalid if some of the assumptions and input data are incorrect [102, 103]. Nonetheless, this quantitative approach could lead to a rational definition of process parameters that would allow rapid optimization of drying kinetics and potentially ensure efficient spray drying process development and scale-up [90]. The ability to predict the performance of new designs or processes before they are even implemented makes computer-aided process design ideally suited to meet the new QbD requirements [104].

9.3.2 The Nano Spray Dryer B-90

The recent rise of nanoparticle-based drug delivery has been the driving force behind pushing the capabilities of conventional spray drying into the nanometer range. Previously, the separation and collection of nanoparticles was extremely difficult due to the low collection efficiency for particles with diameters less than 2 µm [17, 93]. One way to overcome this was to agglomerate nanoparticles into larger microparticles via a two-step approach. This usually involved prior micro-mixing (such as confined liquid impinging jet precipitation, high-gravity controlled precipitation, sonoprecipitation, and multi-inlet vortex mixing) or wet-milling/homogenization to produce a nanosuspension feedstock that would be spray dried [105, 106]. There were, of course, still challenges with adequate re-dispersion of the primary nanoparticles to reap the full benefits of nanosizing.

The Nano Spray Dryer B-90, developed by Büchi, is the newest generation of laboratory-scale spray dryer that incorporates a vibration mesh droplet-generation system, gentle laminar flow heating system, and a highly efficient electrostatic particle collector. As a result, it is now possible to produce particles in the submicron size range (300 nm to 5 µm) with very narrow distributions and high yields [107, 108]. This system is also perfect for early-stage product development in the pharmaceutical and biotechnology industry as

Table 9.2 *Advantages and disadvantages of the Nano Spray Dryer B-90 [109]*

Advantages	Disadvantages
Narrow droplet-size distribution	Expensive
Able to generate particle sizes ranging from 300 nm to 5 μm	Laboratory-scale that has a low throughput and long process time
Suitable for heat-labile therapeutics with minimal loss of activity	Possible clogging of mesh nozzle, which is limited to handling liquid viscosities of up to 10 cps
High yields	Particle size is limited by commercially available nozzle mesh sizes
May utilize sample quantities in milligrams or milliliters	Collected powder resembles a cake that is not free-flowing and requires scraping off using a rubber spatula
Perfect for early-stage product development and drug delivery applications	
Convenient and simplified set up and cleaning procedures	

sample quantities in the milligram or milliliter range can be utilized. Since the novel aspects and limitations of the technology have been reviewed by Heng *et al.* [109], the advantages and disadvantages of the instrument are highlighted in Table 9.2.

Early work with the Nano Spray Dryer focused on demonstrating feasibility and understanding the influence of process parameters on the product. Li *et al.* [107] presented one of the first studies on five polymeric wall materials (arabic gum, whey protein, poly(vinyl alcohol), modified starch, and maltodextrin) and explored the potential to encapsulate nano-emulsions. The study successfully attained nanoparticles down to 350 nm, with yields of 43 to 94.5%. Similarly, Schmid *et al.* [110] evaluated common pharmaceutical excipients (trehalose and mannitol) and the drug griseofulvin. Mean particle diameters between 500 and 800 nm were obtained at reasonably high yields of 50 to 78%. On the other hand, protein nanoparticles of bovine serum albumin (BSA) were optimized by Lee *et al.* [108]. The particles had mean diameters of between 460 and 2609 nm and exhibited four types of particle morphology that consisted of: (i) smooth spherical, (ii) wrinkled, (iii) mixed wrinkled and donut-shaped, and (iv) chips and granules. The particle size was predominantly influenced by the spray mesh aperture, whereas the morphology was affected by the presence of Tween 80. Thus, the study showed that careful tuning of the various process and formulation parameters (via the Taguchi experimental design method) was essential to obtain discrete spherical particles of the desired size. Subsequent work by Bürki *et al.* [111] also used a 3^3 full factorial design to optimize the production of β-galactosidase, with trehalose as a stabilizer. The optimized parameters resulted in a formulation that retained full activity, a particle size of 1.93 μm, and 87% yield.

More recently, other applications of nano-spray-dried formulations have been explored. Studies have exploited the smaller size to enhance dissolution properties of nicergoline [112] and griseofulvin [113], whereas others observed lung targeting of intravenously delivered carboplatin microparticles [114]. Another study reported the first synergistic antimicrobial dry-powder inhaler (DPI) consisting of a binary system of ciprofloxacin

hydrochloride and gatifloxacin hydrochloride [115]. Overall, promising research using the Nano Spray Dryer B-90 has explored a variety of therapeutics including excipients [107], model drugs [107, 110, 112, 113], antibiotics [30, 115], proteins [108, 111], and enzymes [111]. Thus, the timely arrival of the Nano Spray Dryer B-90 offers a new, simple, and alternative method for the development of pharmaceutical powders suitable for a broad range of drug delivery applications.

9.3.3 Novel Multi-Channel Nozzles

The last decade has seen a number of novel multi-channel nozzles coupled to spray drying. Designs have ranged from the standard 2-fluid to multi 3- and 4-fluid constructions, as well as the more recent 2-solution mixing nozzle with customizable configurations. The various multi-channel nozzles and their pharmaceutical applications will be covered here.

The 3-fluid nozzle was first described by Ramtoola [116] as having two concentric channels, which allows the core-forming fluid to flow through the inner stream (containing the active ingredient) and the coat-forming fluid to flow through the outer stream (usually a polymer). Since the 3-fluid nozzle has one atomizing gas channel and two separate liquid channels, the active ingredient and excipients can be dissolved in separate solvents, which eliminates the need for a common solvent [117]. Pabari *et al.* [118] utilized the 3-fluid nozzle to prepare ethyl cellulose-coated diclofenac sodium microparticles. Investigation of the droplet formation from the 3-fluid nozzle, using rhodamine, showed a pink-colored core droplet within a clear polymer coat. In contrast, the droplet formed at the tip of the 2-fluid nozzle was homogeneous and darker in color. Transmission electron microscope images clearly identified a defined core and coat for the 3-fluid nozzle-prepared microparticles, whereas the standard 2-fluid nozzle product showed a homogeneous internal morphology. Hence, this nozzle design is ideal for microencapsulation due to the formation of multilayered droplets [117, 118].

Wan *et al.* [119] attempted to encapsulate lysozyme in poly(lactic-co-glycolic acid) (PLGA) using the 3-fluid nozzle. However, an initial burst effect in the drug release profile (attributed to protein surface enrichment) was a concern as serious toxicity could arise in clinical practice. Later, the same authors incorporated hyaluronic acid to modulate the release profiles of BSA in PLGA [120]. Hyaluronic acid was proposed because the relatively high viscosity of hyaluronic acid even in low concentrations, and the intermolecular entanglement formed between the hyaluronic acid and PLGA molecules, were hypothesized to retard protein migration to the surface. Indeed, the initial burst effect of BSA was suppressed by increasing the amount of hyaluronic acid. Another study, by Kondo *et al.* [121], compared the encapsulation capabilities between the 3- and 4-fluid nozzles using the model drug ethenzamide and ethyl cellulose. In general, the 3-fluid nozzle was found to be superior in creating an effective coat, where slower drug release could be achieved by increasing the concentration of ethyl cellulose. Interestingly, one study has even used the 3-fluid nozzle to generate nanoparticles of isoxyl by antisolvent precipitation [122]. Nanoparticles were formed *in situ* and spray dried immediately to form irregular microparticle aggregates. Table 9.3 provides a summary of pharmaceutical formulations prepared using the 3-fluid nozzle.

Similar to the 3-fluid nozzle, the 4-fluid nozzle consists of two liquid and two atomizing gas channels that can simultaneously spray dry two separate feedstocks. The difference lies

Table 9.3 *Pharmaceutical formulations prepared using 3-fluid nozzle spray drying*

Year	Aqueous	Organic	References
2010	Fish oil	Whey protein	[123]
2010	—	Isoxyl	[122]
2012	Diclofenac sodium	EC	[118]
2012	Rifampicin dihydrate	PLA and PLGA	[124]
2013	Tripolyphosphate	Chitosan	[117]
2013	α-Amylase	Calcium alginate chitosan	[125]
2014	Ethenzamide	EC	[121]
2014	Lysozyme	PLGA	[119]
2014	BSA	PLGA	[120]

BSA: Bovine serum albumin; EC: Ethyl cellulose; PLA: Poly(lactic acid); PLGA: Poly(lactic-co-glycolic acid).

in the acceleration zone where the two liquid channels collide and mix at the tip of the nozzle edge. Ozeki *et al.* [58] demonstrated that polymeric nanoparticles either of ethyl cellulose or PLGA could be dispersed in mannitol microparticles by using 4-fluid nozzle spray drying. The authors proposed that the nanoparticles occurred through antisolvent precipitation and that the mannitol matrix prevented aggregation of the nanoparticles. Pranlukast hemihydrate (PLH) nanoparticles in mannitol microparticles were also produced in the same way [126]. *In vitro* aerosol performance of these PLH-containing microparticles yielded an inhalable fine-particle fraction ($FPF_{5.8\,\mu_m}$) of roughly 25%, and *in vivo* pulmonary administration to rats found that PLH absorption was sustained over a period of 6 h. The authors speculated that the mannitol matrix dissolved immediately after delivery to the lungs, leaving behind the water-insoluble PLH nanoparticles that resulted in prolonged absorption.

Alternatively, inhalable rifampicin-containing mannitol microparticles were prepared by Mizoe *et al.* [127] using the 4-fluid nozzle. Pulmonary delivery to rats found that 86% of the rifampicin could be detected in lung tissue at 5 min, but this reduced to 4% after 1 h. This correlated with plasma concentrations of rifampicin, which peaked at 15 min and returned to 0% at 90 min. In comparison, orally delivered rifampicin was not detectable in lung tissue after 1 h, reaching only approximately 3% after 4 h, whereas intravenously delivered microparticles reached levels of only 0.8% and 1.2% in lung tissue at 1 and 4 h, respectively. Thus, these inhalable microparticles effectively delivered rifampicin to the lungs and, subsequently, systemic circulation. Despite this, methods were required to prolong lung retention and improve targeted drug delivery to alveolar macrophages for TB. As a result, Ohashi *et al.* [128] successfully incorporated PLGA into the formulation to control the release of rifampicin for a period lasting 12 h. Pharmaceutical formulations that have been produced using 4-fluid nozzle spray drying are outlined in Table 9.4.

While the 4-fluid nozzle has been useful, it was unclear whether sufficient mixing had occurred. Therefore, a customizable 2-solution mixing nozzle was developed to enable conditions for optimized mixing [134]. As with both the 3- and 4-fluid nozzles, two separate feedstocks may be spray dried simultaneously. However, this nozzle has a unique internal mixing chamber consisting of four components: injection tubes, mixing joint (bent or linear), mixing tube (swirling or linear), and orifice (0.3 or 0.5 mm). The arrangement of

Table 9.4 *Pharmaceutical formulations produced using 4-fluid nozzle spray drying*

Year	Aqueous	Organic solvent	References
2004	Hydroxypropylmethyl cellulose	Tolubutamide	[129]
2005	Sodium salicylate	Flurbiprofen	[130]
2006	Mannitol	EC or PLGA	[58]
2007	Mannitol or lactose	Ethenzamide or Flurbiprofen	[131]
2007	Mannitol	Pranlukast hemihydrate	[126]
2008	Salbutamol sulfate	EUDRAGIT® RS or EUDRAGIT® RL	[132]
2008	Mannitol	Rifampicin	[127]
2009	Mannitol	Rifampicin and PLGA	[128]
2012	Maltosyl-β-cyclodextrin	ONO-2921 (model drug)	[133]

EC: Ethyl cellulose; PLGA: Poly(lactic-co-glycolic acid).

different configurations affects the flow rate, mixing pattern, and total mixing time. In the case of ethyl cellulose nanoparticles, mixing time could be varied from 0.02 to 3.07 s. Shorter mixing times led to large particle size whilst longer mixing times led to an unchanged size but with a larger standard deviation. To date, ethyl cellulose in mannitol [134] and prubocol with EUDRAGIT® in mannitol [135] microparticles have been generated using this novel 2-solution mixing nozzle.

In summary, these novel multi-channel nozzles can produce multilayered and multifunctional microparticles for drug stabilization, taste masking, controlled-release, and targeted drug delivery. Without a doubt, these nozzles will continue to contribute to the development of innovative formulations in the new decade and beyond.

9.4 Anti-Tuberculosis Therapeutics Produced by Spray Drying

The key advantages proposed for inhaled TB therapy include direct delivery to the site of infection for targeting alveolar macrophages and dendritic cells, reduced overall systemic exposure (and toxicity), and improved patient adherence. More specifically, DPIs offer greater stability, which would be easier and cheaper to administer in developing nations. The following section will evaluate the research that has utilized spray drying to improve various anti-TB therapeutics. The research broadly falls into three categories: (i) controlled-release microparticles incorporating polymers such as PLGA and PLA [124, 128, 136–140], (ii) maximal drug-loaded microparticles with or without excipients [127, 141–145], and (iii) vaccines. Agents that have been incorporated range from the standard first- and second-line drugs to fixed dose combinations, possible adjuncts, and novel anti-TB drug candidates (Table 9.5).

9.4.1 Controlled-Release Microparticles

Pulmonary-delivered formulations tend to have short residence times in the lungs due to the presence of multiple clearance pathways (e.g., cough clearance, mucociliary clearance, macrophage clearance, and systemic absorption). Consequently, synthetic polymers have been extensively used as excipients to control the drug release [137, 165–167]. Biodegradable

Table 9.5 *Anti-tuberculosis formulations produced by spray drying*

Therapeutic class	Active pharmaceutical ingredients	Year	Excipients	Volume median diameter (μm)	Mass median aerodynamic diameter (μm)	Aerosol performance (%)	References
			Controlled-release microparticles				
First-line drugs	Rifampicin	1999	PLA	2.5–6.5	—	—	[139]
		2000	PLGA	2.66–6.84	—	—	[137]
		2008	PLA, PLGA	—	2.22–5.11	$FPF_{5.8\mu m}$ = 22.9–68.4	[136]
		2009	Leucine, PLGA	2.7–4.2	—	$FPF_{5.8\mu m}$ = 35.5–67.8	[140]
		2009	Mannitol, PLGA	—	—	$FPF_{4.7\mu m}$ = 35	[128]
		2012	PLGA, PLA	8.8–24.5	3.5–4.5	$FPF_{5\mu m}$ = 23.9–44.5	[124]
	Isoniazid	2011	Cholesterol, mannitol, PC	—	2.99–4.92	$FPF_{4.4\mu m}$ = 15–30	[146]
	Pyrazinamide	2012	Ammonium carbonate, cholesterol, mannitol, PC	—	4.26–4.39	$FPF_{4.4\mu m}$ = 20–30	[147]
		2013	DPPC, hyaluronic acid and leucine	4.7–26.5	—	—	[148]
Second-line drugs	Levofloxacin	2012	Ammonium carbonate, cholesterol, mannitol, PC	—	4.15–4.44	$FPF_{4.4\mu m}$ = 13–38	[149]
	Ofloxacin	2008	Hyaluronic acid	2–5	—	$FPF_{6.4\mu m}$ = 43	[150]
	Ofloxacin–palladium	2013	PLA	4.6–9	2.5	—	[47]
Fixed dose combinations	Isoniazid and rifabutin	2007	PLA	5.19	3.57	$FPF_{4.6\mu m}$ = 78.91	[151]
	Rifampicin and isoniazid	2013	PLGA, PEG	—	—	—	[152]
Adjunct	Nitric oxide donors (Isosorbide mononitrate, sodium nitroprusside and diethylenetriamine– nitric oxide adduct)	2012	PLGA	4.63–5.78	2.2–2.4	$FPF_{4.6\mu m}$ = 72.4–86.4	[153]

Maximal drug-loaded microparticles							
First-line drugs	Rifampicin	2008	None	6.24–8.25	—	$FPF_{4.7\mu m}$ = 7–47	[145]
		2008	Cholesterol, mannitol, PC	—	—	$FPF_{4.7\mu m}$ = 43	[127]
		2011	None	8.8–24.6	2.2–3.7	$FPF_{5\mu m}$ = 59.1–68.5	[141]
	Rifapentine	2014	None	1.67–1.99	1.68–1.92	$FPF_{5\mu m}$ = 68.8–83.2	[143]
	Isoniazid	2006	Lactose, mannose, trehalose	6.08–6.48	3.14–4.40	$FPF_{5\mu m}$ = 12.0–81.5	[154]
Second-line drugs	Capreomycin	2007	Leucine	4.19	4.99	$FPF_{5.8\mu m}$ = 47.7	[155]
		2008	Leucine	3.14–5.58	4.74–4.99	$FPF_{5.8\mu m}$ = 40.9–52.6	[156]
		2010	Sodium oleate, PLGA	6.69–9.12	—	—	[157]
		2014	None	3.25–3.83	—	$FPF_{6.4\mu m}$ = 27–87	[30]
	p-Aminosalicylic acid	2003	DPPC	7.07–7.47	—	$FPF_{5.6\mu m}$ = 59.6–62.9	[158]
Fixed dose combinations	Isoniazid, pyrazinamide and rifampicin	2013	None	2.4	3.5	$FPF_{5\mu m}$ = 45	[142]
	Moxifloxacin, pyrazinamide and rifapentine	2014	Leucine	1.79–1.82	2.51–2.58	$FPF_{5\mu m}$ = 55.5–63.6	[144]
Adjunct	Pyrazinoate salts	2013	Leucine	—	1.3–5.4	$FPF_{4.46\mu m}$ = 29.7–41	[159]
Novel anti-TB drug candidate	PA-824	2009	DPPC, leucine	4.14	4.74	$FPF_{5.8\mu m}$ = 53.3	[160]
Vaccines							
Vaccines	*Mycobacterium smegmatis* (model of BCG)	2007	Glycerol, leucine	—	3–5	—	[161]
	Ad-35 vectored tuberculosis AERAS-402	2010	Cyclodextrin, dextran, histidine, inositol, leucine, mannitol, PVP, sucrose, trehalose, Tween 80	3.2–3.5	—	—	[162]
Adjuvants	Antigen 85B	2007	PLGA	3.4–4.3	—	—	[163]
	Cutinase-like protein 1 and 6, MPT83	2013	Mannitol	—	—	—	[164]

DPPC: 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine; PC: phosphatidylcholine; PEG: poly(ethylene glycol); PLA: poly(lactic acid); PLGA: poly(lactic-co-glycolic acid); PVP: poly(vinylpyrrolidone).

polymers and co-polymers of lactic and glycolic acids are, by far, the most commonly used because they can degrade under physiological conditions and are authorized as safe for human applications [168]. However, such formulations are yet to appear on the market since issues with long-term safety, in particular the clearance mechanism of high molecular weight polymer from the lungs, remain a concern [169].

9.4.1.1 First-Line Drugs

First-line anti-TB drugs consist of isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin. A majority of the research has centered on spray drying rifamycins (rifampicin, rifabutin, and rifapentine) since they are a family of drugs long considered the mainstay of TB treatment [170, 171]. One of the early studies by Bain *et al.* [139] investigated the fundamental influence of acetonic and halogenated solvents on the characteristics of rifampicin/PLA microparticles. The solubility of the polymer in the solvent directly influenced the drying kinetics and microparticle architecture, as well as the level of residual solvent. That is, particle porosity increased with the rate of polymer deposition, which was linked to lower polymer solubility in a particular solvent. On the other hand, residual solvent increased with polymer solubility and a porous particle generally promoted solvent loss. The study concluded dichloromethane to be the superior solvent to acetone, chloroform, and halothane. Another fundamentally important parameter relating to polymeric particles is particle size. Yoshida *et al.* [172] discovered that size determined phagocytic uptake of PLGA-encapsulated rifampicin. It was found that only 30% of microspheres with diameter 1.5 μm were phagocytosed by alveolar macrophages, compared with 80% of the microspheres with diameter 3.5 μm [172].

In contrast, other studies demonstrated the capabilities of spray drying to produce polymeric microparticles. O'Hara and Hickey [137] compared PLGA-encapsulated rifampicin microparticles prepared by spray drying versus those prepared by traditional solvent evaporation. Spray-dried powders achieved superior drug loading efficiency, and subsequently higher average percentage of drug release since the drug could not partition into an external phase (as is the case with solvent evaporation). Based on a volume median diameter (VMD) of 2.66 to 2.76 μm alone, the authors proposed that aerosol delivery of the spray-dried powder should be effective, although no firm conclusion could be drawn. The work was continued to *in vivo* studies by Suarez *et al.* [173], who administered the spray-dried formulations to *M. tuberculosis*-infected guinea pigs. The PLGA-encapsulated rifampicin microparticles led to a significantly reduced number of viable bacteria, as well as less inflammation and lung damage compared with rifampicin-only or control-treated animals. These preliminary results supported the potential of inhalable PLGA-encapsulated rifampicin microparticles for TB.

Sung *et al.* [140] also carried out a two-step solvent evaporation followed by spray drying process to encapsulate rifampicin into PLGA nanoparticles. This obtained porous nanoparticle aggregates that were suitable for inhalation. Pulmonary delivery to healthy guinea pigs achieved higher systemic bioavailability than did the oral route, and led to extended levels of drug in lung tissue up to and beyond 8 h. On the other hand, Coowanitwong *et al.* [136] co-spray dried rifampicin with PLA or PLGA. These microparticles had mass median aerodynamic diameters (MMAD) ranging from 2.22 to 5.11 μm and fine-particle fractions less than 5.8 μm ($FPF_{5.8\mu m}$) ranging from 22.9 to 68.4%, with PLA-encapsulated microspheres performing better.

In addition to the rifamycins, pyrazinamide and isoniazid have also been studied. Rojanarat *et al.* [146, 147] prepared proliposome powders containing either isoniazid or pyrazinamide. The obtained microparticles had encapsulation efficiencies of 18–30% and 26–45% for isoniazid and pyrazinamide, respectively, and were suitable for inhalation with MMAD 2.99–4.92 µm (isoniazid) and 4.26–4.39 µm (pyrazinamide).

9.4.1.2 Second-Line Drugs

Second-line, or reserve, drugs are the last line of defence against drug-resistant TB. This group features agents which are more expensive, more toxic, and less effective than standard treatments [171, 174]. Most of these proof-of-concept studies simply tested the feasibility of producing an inhaled formulation by spray drying drugs that included levofloxacin and ofloxacin. Rojanarat *et al.* [149] prepared levofloxacin proliposomes, whereas Hwang *et al.* [150] co-spray dried ofloxacin with sodium salt and hyaluronic acid. Palazzo *et al.* [47] further optimized a PLA-encapsulated ofloxacin–palladium complex using design of experiments.

9.4.1.3 Fixed Dose Combinations

The World Health Organization recommends the use of fixed dose combinations for two main reasons: (i) to prevent emergence of drug-resistant strains that occur from monotherapy, and (ii) to reduce prescription errors because dosage recommendations are more straightforward and adjustment of dosage according to patient weight would be easier [171]. Furthermore, this can potentially translate into measurable improvements in patient adherence.

Co-spray drying (from a solution, suspension, or even a mixture of the two) can produce DPI combinations in a single, industrially scalable process. Muttil *et al.* [151] was the first to co-spray dry isoniazid and rifabutin, with and without PLA. Rifabutin was chosen because rifampicin has the potential to cause stability-related problems [165]. Previous rifampicin experiments also exhibited chemical incompatibility with isoniazid in organic solutions [167, 175]. Later, Booysen *et al.* [152] prepared PLGA-nanoparticles incorporating both rifampicin and isoniazid, which were also coated with poly(ethylene glycol). These nanoparticles demonstrated sustained drug release over seven days that could potentially reduce the dosing frequencies of rifampicin and isoniazid.

Even though co-spray drying has become a popular technique, innovative methods are needed to address problems with stability and segregation of components (as well as homogeneity and reproducible delivery of each drug) over time [144]. Since different drugs in the single particle may have vastly different physicochemical properties, the formulation conditions that maximize stability for one drug may adversely impact the stability of another [176]. In addition, the development of new analytical methods for combination products may present significant challenges [169].

9.4.1.4 Adjunct – Nitric Oxide

Possible adjuncts as add-on therapy to the standard TB regimen may include controlled-release nitric oxide donors. One mechanism that *M. tuberculosis* uses to evade the innate immune response is through the inhibition of nitric oxide production inside macrophages [177]. Consequently, Verma *et al.* [153] investigated the impact of nitric oxide donors on

the bacterial burden *in vitro* of *M. tuberculosis*-infected macrophages. Three clinically proven nitric oxide donors (isosorbide mononitrate, sodium nitroprusside, and diethylenetriamine–nitric oxide adduct) were separately co-spray dried with PLGA. Encapsulation efficiencies greater than 90% were achieved and MMAD ranged from 2.2 to 2.4 μm. These microparticles were observed to induce a significant dose-dependent bactericidal activity, compared to that shown by equivalent amounts of drug in solution. The study also reported a descending trend of bactericidal activity that followed the order: diethylenetriamine–nitric oxide adduct > sodium nitroprusside > isosorbide mononitrate. Overall, these findings suggest that nitric oxide donors could be useful as adjuncts to enhance the bactericidal activity of other agents against TB.

9.4.2 Maximal Drug-loaded Microparticles

Apart from controlled-release polymeric microparticles, maximal drug-loaded microparticles (with or without excipients) have also been of interest to deliver higher local drug concentrations to the lungs.

9.4.2.1 First-Line Drugs

Fundamental studies have found that the selection of solvent for spray drying had a role in controlling the physicochemical properties and aerosolization behavior of excipient-free rifampicin microparticles [145, 178]. Moreover, the main limitation associated with spray drying is that most materials undergo amorphization, which can become a stability issue. Amorphous spray-dried rifampicin has been reported to undergo significant chemical degradation after 9 months, losing 26% of its chemical potency [141]. As a result, Son *et al.* [141] described a process for the polymorphic transformation of rifampicin by recrystallization of pure rifampicin in anhydrous ethanol. The crystallized suspension was homogenized and spray dried to produce flake-like agglomerates of rifampicin dihydrate microparticles. The unique particle morphology led to improved aerosolization properties over those shown by amorphous spherical rifampicin particles. Furthermore, significant chemical stability (only 3% reduction in chemical potency) was observed. Similarly, Chan *et al.* [143] developed a novel inhalable crystalline form of rifapentine suitable for targeted treatment of TB that has the potential to radically shorten treatment times.

Other less extensively investigated first-line agents incorporating excipients have included isoniazid and pyrazinamide. These studies generally determined the feasibility of spray drying an inhalable formulation. Sawatdee *et al.* [154] co-spray dried isoniazid with sugars (trehalose, mannose, and lactose), whereas Pham *et al.* [148] attempted to enhance the stability of spray-dried pyrazinamide through the use dipalmitoylphosphatidycholine (DPPC), hyaluronic acid, and leucine.

9.4.2.2 Second-Line Drugs

Different investigators have spray dried inhalable capreomycin powders [30, 155–157]. Schoubben *et al.* [157] ion-paired capreomycin with sodium oleate and prepared microparticles by homogenization followed by spray drying. Qualitatively, the powders had a suitable size (VMD of 6.18 μm) and shape for inhalation. Later, the same group also spray dried pure capreomycin powders using the Nano Spray Dryer B-90 [30]. A 2^3 factorial

design was used to optimize the process parameters to obtain inhalable capreomycin powders with $FPF_{6.4\mu m}$ values ranging from 9 to 26%.

Alternatively, other authors co-spray dried capreomycin solutions with leucine as the main excipient [155, 156]. The powders possessed good aerosolization properties and physical-chemical stability for up to 3 months at room temperature. Pulmonary delivery to healthy guinea pigs showed significantly lower systemic exposure and longer drug half-life than did the intravenous or intramuscular routes [156], whereas inhalation by *M. tuberculosis*-infected guinea pigs resulted in significantly lower levels of inflammation, bacterial burden, and percentage of lung tissue affected by granulomas and caseous necrosis [155]. Also, sequential doses did not accumulate in the lungs of healthy guinea pigs and achieved higher local lung concentrations at all times [179]. Recently, a Phase I clinical trial confirmed that inhaled doses of spray-dried capreomycin was well tolerated in humans [180].

Tsapis *et al.* [158] also prepared dry powders of *p*-aminosalicylic acid by spray drying a solution containing DPPC as an excipient. Large porous particles had VMD values of 7.07–7.47 μm, contained drug loading of 95% by weight, and displayed physical stability over 4 weeks at elevated temperatures.

9.4.2.3 Fixed Dose Combinations

Recently, two triple-combinations of anti-TB drugs were reported by Chan *et al.* [142, 144]. The excipient-free formulation of pyrazinamide/rifampicin/isoniazid had an MMAD of 3.5 μm and $FPF_{5\mu m}$ of 45% [142]. Unfortunately, the chemical incompatibility between rifampicin and isoniazid was not taken into consideration, as happened also for the study by Booysen *et al.* [152]. Consequently, a new triple-combination of moxifloxacin/pyrazinamide/rifapentine (with or without leucine as an excipient) was devised. The formulation was suitable for inhalation, with an MMAD ranging from 2.51 to 2.58 μm and an $FPF_{5\mu m}$ of 55–64% [144].

9.4.2.4 Adjunct – Pyrazinoate Salts

According to Zhang and Mitchison [181], pyrazinamide is a pro-drug which is metabolized by the liver to the active moiety – pyrazinoic acid (POA). The bactericidal activity of POA is greatly dependent on pH such that small changes towards increased acidity could be expected to enhance the efficacy of pyrazinamide against *M. tuberculosis.* Mitchison and Fourie [1] proposed that inhaled POA could be a suitable adjunct to oral pyrazinamide. This should be able to induce the required acidic shift in pH within tuberculosis lesions, thereby potentially reducing the oral dose of pyrazinamide and hepatotoxic side effects. Durham *et al.* [159] spray dried pyrazinoate salts to achieve MMAD values down to 1.3 μm and $FPF_{4.46\mu m}$ values up to 41%. The powders had particle sizes and morphology suitable for pulmonary delivery, but further work is needed to obtain a unimodal aerodynamic particle-size distribution and increase the fine particle fraction.

9.4.2.5 Novel Anti-TB Drug Candidate

PA-824 is a novel anti-TB candidate for drug-resistant TB [182]. Phase 2 clinical trials have been completed and plans to start a Phase 3 trial are underway [183]. Sung *et al.* [160] investigated the feasibility of preparing an aerosol formulation of PA-824. The spray-dried

microparticles, with leucine as an excipient, were porous and had desirable aerosol properties (MMAD 4.74 μm and $FPF_{5.8\mu m}$ 53.3%). The powder was physically, aerodynamically, and chemically stable for 6 months at room temperature. Pulmonary delivery to healthy guinea pigs resulted in sustained levels in the lung for 32 h post-exposure, whereas pulmonary administration to TB-infected guinea pigs showed significantly less inflammation, viable bacteria count, and tissue damage compared with results in untreated animals [184]. However, no difference in tissue damage was observed between groups that received either the oral or inhaled dose. Therefore, these studies only indicate the potential use of PA-824 as a DPI in the treatment of TB.

9.4.3 Vaccines

Inhalable DPI vaccines are envisioned as needle-free alternatives with better room temperature stability, which would be easier to administer in rural areas that have limited resources such as access to sterile water [14, 15]. Recent studies have reported spray drying inhalable Ad35-vectored tuberculosis (AERAS-402) [162] and Bacillus Calmette-Guérin (BCG) vaccines as improvements to the traditional injection. Wong *et al.* [161] successfully prepared a dry, flowable powder of BCG that was more viable and more stable for several months than was the conventional lyophilized formulation. The authors found that leucine and glycerol were crucial excipients that protected bacterial viability by limiting the osmotic stress on bacterial membranes during the spray drying process. Consequently, the spray-dried powders retained 48% viability, compared with the conventionally lyophilized sample that retained only 3.8% viability. Garcia-Contreras *et al.* [185] later administered the DPI vaccine as aerosols to guinea pigs, which were subsequently challenged with virulent *M. tuberculosis*. The study observed significantly reduced bacterial burden and lung pathology relative to both untreated animals and control animals (immunized with the standard parenteral vaccine). Despite the exciting opportunities offered by inhalable vaccines, several issues concerning the risk of hypersensitivity or intolerance to inhaled vaccines must be addressed prior to their introduction into clinical practice [15, 186, 187].

Proteins secreted by *M. tuberculosis* [such as antigen 85B, cutinase-like proteins (Culp) 1 and 6 and MPT83] are possible adjuvants for inhalable TB vaccines [164, 188]. These agents may be beneficial to enhance the efficacy of subunit vaccines as they often have weaker antigenicity and immunogenicity [189–192]. Lu *et al.* [163] encapsulated antigen 85B into PLGA microspheres and found the adjuvant elicited higher *in vitro* T cell activation that targeted alveolar macrophages more efficiently. In addition, the other observed advantage was sustained release over six days. In comparison, Tyne *et al.* [164] investigated Culp 1 and 6 and MPT83 conjugated directly to the novel adjuvant Lipokel (Lipotek Pty Ltd). Protein–Lipokel complexes were formulated as dry powders for pulmonary delivery by spray drying with mannitol. Direct delivery to the lungs of mice led to recruitment of neutrophils and antigen-presenting cells that persisted for 7 days post-immunization. Notably, pulmonary immunization with either Culp1-6–Lipokel or MPT83–Lipokel powder vaccines generated protective responses in the lungs against *M. tuberculosis* challenge. Hence, these preliminary works present the foundation for further investigations of proteins secreted by *M. tuberculosis* as adjuvants that aid the development of easily administrable and protective vaccines against TB.

9.5 Conclusion

In conclusion, this chapter has critically reviewed the extensive research related to spray drying drug delivery systems for the treatment and prevention of TB. There is a promising pipeline emerging through the re-purposing of old drugs, re-engineering of existing agents, and discovery of new compounds [2, 193–201]. Although significant advances have been made, these promising therapies are yet to be translated into clinical practice [11, 202].

Spray-dried combinations of new and existing drugs have been explored to shorten treatment duration, simplify therapy, and treat drug-resistant TB. While existing research will continue on controlled-release and maximal drug-loaded microparticles, a future trend could see increased investigations into synergistic combinations [115, 203]. Synergistic combinations should accelerate the eradication of TB and simplify treatment regimens, further improving patient adherence and reducing the emergence of drug-resistant TB. It is essential that the fundamental work reported to date progresses through to human clinical trials, and beyond, in order to truly revolutionize TB treatment. Nonetheless, spray drying and its recent technological advances are well positioned to provide the solutions to stop TB. The future looks bright for engineered particles and continued advances will pave the way towards targeted delivery of therapeutics.

9.6 Acknowledgements

Jennifer Wong was a recipient of the Endeavour Research Fellowship from the Department of Education and Training, Australia. This work was supported under the Australian Research Council's *Discovery Projects* funding scheme (projects DP120102778 & 110105161).

References

[1] Mitchison, D.A. and Fourie, P.B. (2010) The near future: Improving the activity of rifamycins and pyrazinamide. *Tuberculosis*, **90**(3), 177–181.
[2] Zumla, A.I., Gillespie, S.H., Hoelscher, M. *et al.* (2014) New antituberculosis drugs, regimens, and adjunct therapies: Needs, advances, and future prospects. *Lancet Infect. Dis.*, **14**(4), 327–340.
[3] Sosnik, A., Carcaboso, Á.M., Glisoni, R.J. *et al.* (2010) New old challenges in tuberculosis: Potentially effective nanotechnologies in drug delivery. *Adv. Drug Deliv. Rev.*, **62**(4–5), 547–559.
[4] Blasi, P., Schoubben, A., Giovagnoli, S. *et al.* (2009) Fighting tuberculosis: Old drugs, new formulations. *Expert Opin. Drug Deliv.*, **6**(9), 977–993.
[5] Gupta, A., Pandya, S.M., Mohammad, I. *et al.* (2013) A. Particulate pulmonary delivery systems containing anti-tuberculosis agents. *Crit. Rev. Ther. Drug Carrier Syst.*, **30**(4), 277–291.
[6] Misra, A., Hickey, A.J., Rossi, C. *et al.* (2011) Inhaled drug therapy for treatment of tuberculosis. *Tuberculosis*, **91**(1), 71–81.
[7] González-Juarrero, M. and O'Sullivan, M.P. (2011) Optimization of inhaled therapies for tuberculosis: The role of macrophages and dendritic cells. *Tuberculosis*, **91**(1), 86–92.
[8] Verma, R.K., Singh, A.K., Mohan, M. *et al.* (2011) Inhaled therapies for tuberculosis and the relevance of activation of lung macrophages by particulate drug-delivery systems. *Ther. Deliv.*, **2**(6), 753–768.
[9] Chan, J.G.Y., Bai, X. and Traini, D. (2014) An update on the use of rifapentine for tuberculosis therapy. *Expert Opin. Drug Deliv.*, **11**(3), 421–431.

[10] Hoppentocht, M., Hagedoorn, P., Frijlink, H.W. and de Boer, A.H. (2014) Developments and strategies for inhaled antibiotic drugs in tuberculosis therapy: A critical evaluation. *Eur. J. Pharm. Biopharm.*, **86**(1), 23–30.
[11] Muttil, P., Wang, C. and Hickey, A.J. (2009) Inhaled drug delivery for tuberculosis therapy. *Pharm. Res.*, **26**(11), 2401–2416.
[12] Pham, D.-D., Fattal, E. and Tsapis, N. (2015) Pulmonary drug delivery systems for tuberculosis treatment. *Int. J. Pharm.*, **478**(2), 517–529.
[13] Weers, J.G., Tarara, T.E. and Clark, A.R. (2007) Design of fine particles for pulmonary drug delivery. *Expert Opin. Drug Deliv.*, **4**(3), 297–313.
[14] Fourie, P., Germishuizen, W., Wong, Y.-L. and Edwards, D. (2008) Spray drying TB vaccines for pulmonary administration. *Expert Opin. Biol. Ther.*, **8**(7), 857–863.
[15] Sou, T., Meeusen, E.N., de Veer, M. et al. (2011) New developments in dry powder pulmonary vaccine delivery. *Trends Biotechnol.*, **29**(4), 191–198.
[16] Chow, A.H.L., Tong, H.H.Y., Chattopadhyay, P. and Shekunov, B.Y. (2007) Particle engineering for pulmonary drug delivery. *Pharm. Res.*, **24**(3), 411–437.
[17] Vehring, R. (2008) Pharmaceutical particle engineering via spray drying. *Pharm. Res.*, **25**(5), 999–1022.
[18] Patel, B.B., Patel, J. and Chakraborty, S. (2014) Review of patents and application of spray drying in pharmaceutical, food and flavor industry. *Recent Pat. Drug Deliv. Formul.*, **8**(1), 63–78.
[19] Cipolla, D.C. and Gonda, I. (2011) Formulation technology to repurpose drugs for inhalation delivery. *Drug Discov. Today: Ther. Strateg.*, **8**(3–4), 123–130.
[20] Percy, S.R. (1872) Improvement in drying and concentrating liquid substances by atomizing. US Patent 125406, 9/4/1872.
[21] Merrell, I.S. (1915) Method of desiccating. US Patent 1133051 A.
[22] Wells, L. (1912) *Condensed and dessicated milk*. United States Department of Agriculture Yearbook, pp. 335–344.
[23] Humbert, S., Loerincik, Y., Rossi, V. *et al.* (2009) Life cycle assessment of spray dried soluble coffee and comparison with alternatives (drip filter and capsule espresso). *J. Clean. Prod.*, **17**(15), 1351–1358.
[24] Blouin, J.J. (inventor); W.R. Grace & Co.-Conn. (New York, NY) (assignee). Spray dried water dispersible fertilizer. US Patent 5435822, 25/7/1995.
[25] Gharsallaoui, A., Roudaut, G., Chambin, O. *et al.* (2007) Applications of spray-drying in microencapsulation of food ingredients: An overview. *Food Res. Int.*, **40**(9), 1107–1121.
[26] Feng, T., Xiao, Z. and Tian, H. (2009) Recent patents in flavor microencapsulation. *Recent Pat. Food Nutr. Agric.*, **1**(3), 193–202.
[27] Feng, T., Xiao, Z. and Tian, H. (2010) Recent patents on nano flavor preparation and its application. *Recent Pat. Food Nutr. Agric.*, **2**(3), 243–250.
[28] Estevinho, B.M.A.N., Rocha, F.A.N., Santos, L.M.D.S. and Alves, M.A.C. (2013) Using water-soluble chitosan for flavour microencapsulation in food industry. *J. Microencapsul.*, **30**(6), 571–579.
[29] Charve, J. and Reineccius, G.A. (2009) Encapsulation performance of proteins and traditional materials for spray dried flavors. *J. Agric. Food Chem.*, **57**(6), 2486–2492.
[30] Schoubben, A., Giovagnolim S., Tiralti, M.C. *et al.* (2014) Capreomycin inhalable powders prepared with an innovative spray-drying technique. *Int. J. Pharm.*, **469**(1), 132–139.
[31] Schoubben, A., Blasi, P., Marenzoni, M.L. *et al.* (2013) Capreomycin supergenerics for pulmonary tuberculosis treatment: Preparation, in vitro, and in vivo characterization. *Eur. J. Pharm. Biopharm.*, **83**(3), 388–395.
[32] Cho, W., Kim, M.-S., Jung, M.-S. *et al.* (2015) Design of salmon calcitonin particles for nasal delivery using spray-drying and novel supercritical fluid-assisted spray-drying processes. *Int. J. Pharm.*, **478**(1), 288–296.
[33] Chen, K.H., Di Sabatino, M., Albertini, B. *et al.* (2013) The effect of polymer coatings on physicochemical properties of spray-dried liposomes for nasal delivery of BSA. *Eur. J. Pharm. Sci.*, **50**(3–4), 312–322.
[34] Wang, S.H., Kirwan, S.M., Abraham, S.N. *et al.* (2012) Stable dry powder formulation for nasal delivery of anthrax vaccine. *J. Pharm. Sci.*, **101**(1), 31–47.

[35] Garmise, R.J., Staats, H.F. and Hickey, A.J. (2007) Novel dry powder preparations of whole inactivated influenza virus for nasal vaccination. *AAPS PharmSciTech.*, **8**(4), 2–10.
[36] Bowey, K. and Neufeld, R. (2010) Systemic and mucosal delivery of drugs within polymeric microparticles produced by spray drying. *BioDrugs*, **24**(6), 359–377.
[37] Mortensen, N.P., Durham, P. and Hickey, A.J. (2014) The role of particle physico-chemical properties in pulmonary drug delivery for tuberculosis therapy. *J. Microencapsul.*, **31**(8), 785–795.
[38] Hanif, S.N.M. and Garcia-Contreras, L. (2012) Pharmaceutical aerosols for the treatment and prevention of tuberculosis. *Front. Cell. Infect. Microbiol.*, **2**, 118.
[39] Weers, J. and Tarara, T. (2014) The PulmoSphere™ platform for pulmonary drug delivery. *Ther. Deliv.*, **5**(3), 277–295.
[40] Geller, D.E., Weersm, J. and Heuerding, S. (2011) Development of an inhaled dry-powder formulation of tobramycin using PulmoSphere™ technology. *J. Aerosol Med. Pulm. Drug Deliv.*, **24**(4), 175–182.
[41] Pilcer, G. and Amighi, K. (2010) Formulation strategy and use of excipients in pulmonary drug delivery. *Int. J. Pharm.*, **392**(1–2), 1–19.
[42] Seville, P.C., Li, H.-y. and Learoyd, T.P. (2007) Spray-dried powders for pulmonary drug delivery. *Crit. Rev. Ther. Drug Carrier Syst.*, **24**(4), 307–360.
[43] Yu, M., Zhou, K., Li, Z. and Zhang, D. (2014) Preparation, characterization and in vitro gentamicin release of porous HA microspheres. *Mater. Sci. Eng. C Mater. Biol. Appl.*, **45**, 306–312.
[44] Fernández-Olleros, A.M., Olmo, R., Muñíz, E. *et al.* (2014) In-vivo evaluation of tamoxifen-loaded microspheres based on mixtures of poly (d,l-lactide-co-glycolide) and poly (d,l-lactide) polymers. *Anticancer Drugs*, **25**, 641–651.
[45] Xu, F.H. and Zhang, Q. (2007) Recent advances in the preparation progress of protein/peptide drug loaded PLA/PLGA microspheres. *Yao Xue Xue Bao*, **42**, 1–7.
[46] Alpar, H.O., Somavarapu, S., Atuah, K.N. and Bramwell, V.W. (2005) Biodegradable mucoadhesive particulates for nasal and pulmonary antigen and DNA delivery. *Adv. Drug Deliv. Rev.*, **57**(3), 411–430.
[47] Palazzo, F., Giovagnoli, S., Schoubben, A. *et al.* (2013) Development of a spray-drying method for the formulation of respirable microparticles containing ofloxacin–palladium complex. *Int. J. Pharm.*, **440**(2), 273–282.
[48] Weisheimer, V., Miron, D., Silva, C.B. *et al.* (2010) Microparticles containing lemongrass volatile oil: Preparation, characterization and thermal stability. *Pharmazie*, **65**(12), 885–890.
[49] De Koker, S., Fierens, K., Dierendonck, M. *et al.* (2014) Nanoporous polyelectrolyte vaccine microcarriers. A formulation platform for enhancing humoral and cellular immune responses. *J. Control. Release*, **195**, 99–109.
[50] Zhu, C., Shoji, Y., McCray, S. *et al.* (2014) Stabilization of HAC1 influenza vaccine by spray drying: Formulation development and process scale-up. *Pharm. Res.*, **31**(11), 3006–3018.
[51] McAdams, D., Chen, D. and Kristensen, D. (2012) Spray drying and vaccine stabilization. *Expert Rev. Vaccines*, **11**(10), 1211–1219.
[52] Gao, P. (2008) Amorphous pharmaceutical solids: Characterization, stabilization, and development of marketable formulations of poorly soluble drugs with improved oral absorption. *Mol. Pharm.*, **5**(6), 903–904.
[53] Dhumal, R.S., Biradar, S.V., Aher, S. and Paradkar, A.R. (2009) Cefuroxime axetil solid dispersion with polyglycolized glycerides for improved stability and bioavailability. *J. Pharm. Pharmacol.*, **61**(6), 743–751.
[54] Niwa, T., Miura, S. and Danjo, K. (2011) Design of dry nanosuspension with highly spontaneous dispersible characteristics to develop solubilized formulation for poorly water-soluble drugs. *Pharm. Res.*, **28**(9), 2339–2349.
[55] Paudel, A., Worku, Z.A., Meeus, J. *et al.* (2013) Manufacturing of solid dispersions of poorly water soluble drugs by spray drying: Formulation and process considerations. *Int. J. Pharm.*, **453**(1), 253–284.
[56] Masters, K. (2002) *Spray drying in practice*. SprayDryConsult International, Charlottenlund, Denmark.
[57] Chan, H.-K. and Chew, N.Y.K. (2003) Novel alternative methods for the delivery of drugs for the treatment of asthma. *Adv. Drug Deliv. Rev.*, **55**(7), 793–805.

[58] Ozeki, T., Beppu, S., Mizoe, T. *et al.* (2006) Preparation of polymeric submicron particle-containing microparticles using a 4-fluid nozzle spray drier. *Pharm. Res.*, **23**(1), 177–183.
[59] Masters, K. (1991) *Spray Drying Handbook*, 5th edn. Wiley & Sons, Inc., New York NY.
[60] Huang, L.X. and Mujumdar, A.S. (2008) The effect of rotary disk atomizer RPM on particle size distribution in a semi-industrial spray dryer. *Dry. Technol.*, **26**(11), 1319–1325.
[61] Mujumdar, A.S. (2006) *Handbook of Industrial Drying*, 3rd edn., CRC Press, Boca Raton, FL.
[62] Barbosa-Cánovas, G.V., Ortega-Rivas, E., Juliano, P. and Yan, H. (2005) Drying. In: Barbosa-Cánovas, G.V. (ed.), *Food Powders, Physical Properties, Processing, and Functionality*, Food Engineering Series, Kluwer Academic/Plenum Publishers, New York, NY.
[63] van't Land, C.M. (2011) Spray Drying. In: van't Land, C.M. (ed.), *Drying in the Process Industry*, John Wiley & Sons, Inc., New Jersey, pp. 133–162.
[64] Hickey, A.J., Concessio, N.M., Van Oort, M.M. and Platz, R.M. (1994) Factors influencing the dispersion of dry powders as aerosols. *Pharm. Technol.*, **18**(8), 58–64.
[65] Broadhead, J., Edmond Rouan, S.K., Hau, I. and Rhodes, C.T. (1994) The effect of process and formulation variables on the properties of spray dried b-galactosidase. *J. Pharm. Pharmacol.*, **46**, 458–467.
[66] Maa, Y.-F., Nguyen, P.-A., Sit, K. and Hsu, C.C. (1998) Spray-drying performance of a bench-top spray dryer for protein aerosol powder preparation. *Biotechnol. Bioeng.*, **60**(3), 301–309.
[67] Masters, K. (2004) Applying spray drying to customised powder manufacture. *Chin. J. Chem. Eng.*, **12**, 744–749.
[68] Getler, J.L. (1990) Spray drying of bioproducts. In: Kadam, K.L. (ed.), *Granulation Technology for Bioproducts*, CRC Press, Boca Raton, FL.
[69] Freitas, S., Merkle, H.P. and Gander, B. (2004) Ultrasonic atomisation into reduced pressure atmosphere—envisaging aseptic spray-drying for microencapsulation. *J. Control. Release*, **95**(2), 185–195.
[70] Giovagnoli, S., Palazzo, F., Michele, A.D. *et al.* (2014) The influence of feedstock and process variables on the encapsulation of drug suspensions by spray-drying in fast drying regime: The case of novel antitubercular drug–palladium complex containing polymeric microparticles. *J. Pharm. Sci.*, **103**(4), 1255–1268.
[71] Sloth, J., Jørgensen, K., Bach, P. *et al.* (2009) Spray drying of suspensions for pharma and bio products: Drying kinetics and morphology. *Ind. Eng. Chem. Res.*, **48**(7), 3657–3664.
[72] Maury, M., Murphy, K., Kumar, S. *et al*. (2005) Effects of process variables on the powder yield of spray-dried trehalose on a laboratory spray-dryer. *Eur. J. Pharm. Biopharm.*, **59**(3), 565–573.
[73] Vehring, R., Foss, W.R. and Lechuga-Ballesteros, D. (2007) Particle formation in spray drying. *J. Aerosol Sci.*, **38**(7), 728–746.
[74] Nandiyanto, A.B.D. and Okuyama, K. (2011) Progress in developing spray-drying methods for the production of controlled morphology particles: From the nanometer to submicrometer size ranges. *Adv. Powder Technol.*, **22**(1), 1–19.
[75] Oakley, D.E. (2004) Spray dryer modeling in theory and practice. *Drying Technol.*, **22**(6), 1371–1402.
[76] Langrish, T.A.G. and Fletcher, D.F. (2003) Prospects for the modelling and design of spray dryers in the 21st century. *Drying Technol.*, **21**(2), 197–215.
[77] Boraey, M.A. and Vehring, R. (2014) Diffusion controlled formation of microparticles. *J. Aerosol Sci.*, **67**, 131–143.
[78] Zbicinski, I., Delag, A., Strumillo, C. and Adamiec, J. (2002) Advanced experimental analysis of drying kinetics in spray drying. *Chem. Eng. J.*, **86**(1–2), 207–216.
[79] Hecht, J.P. and Bayly, A.E. (2009) *Atomization for spray drying: Unanswered questions and industrial needs*. ICLASS 2009 11th Triennial International Annual Conference on Liquid Atomization and Spray Systems, Vail, Colorado, USA.
[80] Farid, M. (2003) A new approach to modelling of single droplet drying. *Chem. Eng. Sci.*, **58**(13), 2985–2993.
[81] Bahadur, J., Sen, D., Mazumder, S. *et al.* (2010) Evaporation-induced self assembly of nanoparticles in non-buckling regime: Volume fraction dependent packing. *J. Colloid Interf. Sci.*, **351**(2), 357–364.

[82] Huang, D. (2011) *Modeling of particle formation during spray drying.* III European Drying Conference EuroDrying, 26–28 October, Palma, Spain.
[83] Kuts, P.S., Strumillo, C. and Zbicinski, I. (1996) Evaporation kinetics of single droplets containing dissolved bioaiass. *Drying Technol.*, **14**(9), 2041–2060.
[84] Nešić, S. and Vodnik, J. (1991) Kinetics of droplet evaporation. *Chem. Eng. Sci.*, **46**(2), 527–537.
[85] Charlesworth, D.H. and Marshall, W.R. (1960) Evaporation from drops containing dissolved solids. *AIChE J.*, **6**(1), 9–23.
[86] Adler, M. and Lee, G. (1999) Stability and surface activity of lactate dehydrogenase in spray-dried trehalose. *J. Pharm. Sci.*, **88**(2), 199–208.
[87] Thybo, P., Hovgaard, L., Lindeløv, J. *et al.* (2008) Scaling up the spray drying process from pilot to production scale using an atomized droplet size criterion. *Pharm. Res.*, **25**(7), 1610–1620.
[88] Yu, L., Amidon, G., Khan, M. *et al*. (2014) Understanding pharmaceutical quality by design. *AAPS J.*, **16**(4), 771–783.
[89] US FDA. Guidance for Industry: Q8(R2) Pharmaceutical Development: U.S. Department of Health and Human Services Food and Drug Administration November 2009 [3/2/2015]. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatory Information/Guidances/UCM073507.pdf. (Accessed 20th March 2016.)
[90] Dobry, D.E., Settell, D.M., Baumann, J.M. *et al.* (2009) A model-based methodology for spray-drying process development. *J. Pharm. Innov.*, **4**(3), 133–142.
[91] Maltesen, M.J., Bjerregaard, S., Hovgaard, L. *et al.* (2008) Quality by design – spray drying of insulin intended for inhalation. *Eur. J. Pharm. Biopharm.*, **70**(3), 828–838.
[92] Baldinger, A., Clerdent, L., Rantanen, J. *et al.* (2012) Quality by design approach in the optimization of the spray-drying process. *Pharm. Dev.Technol*., **17**(4), 389–397.
[93] Prinn, K., Costantino, H. and Tracy, M. (2002) Statistical modeling of protein spray drying at the lab scale. *AAPS PharmSciTech.*, **3**(1), 32–39.
[94] Billon, A., Bataille, B., Cassanas, G. and Jacob, M. (2000) Development of spray-dried acetaminophen microparticles using experimental designs. *Int. J. Pharm.*, **203**(1–2), 159–168.
[95] Lebrun, P., Krier, F., Mantanus, J. *et al*. (2012) Design space approach in the optimization of the spray-drying process. *Eur. J. Pharm. Biopharm.*, **80**(1), 226–234.
[96] Ståhl, K., Claesson, M., Lilliehorn, P. *et al.* (2002) The effect of process variables on the degradation and physical properties of spray dried insulin intended for inhalation. *Int. J. Pharm.*, **233**(1–2), 227–237.
[97] Gonnissen, Y., Gonçalves, S.I.V., De Geest, B.G. *et al*. (2008) Process design applied to optimise a directly compressible powder produced via a continuous manufacturing process. *Eur. J. Pharm. Biopharm.*, **68**(3), 760–770.
[98] Silva, A.S., Tavares, M. and Aguiar-Ricardo, A. (2014) Sustainable strategies for nano-in-micro particle engineering for pulmonary delivery. *J. Nanopart. Res.*, **16**(11), 1–17.
[99] Gac, J.M. and Gradoń, L. (2013) A distributed parameter model for the spray drying of multicomponent droplets with a crust formation. *Adv. Powder Technol.*, **24**(1), 324–330.
[100] Tsapis, N., Bennett, D., Jackson, B. et al. (2002) Trojan particles: Large porous carriers of nanoparticles for drug delivery. *Proc. Natl. Acad. Sci.*, **99**(19), 12001–12005.
[101] Vicente, J., Pinto, J., Menezes, J. and Gaspar, F. (2013) Fundamental analysis of particle formation in spray drying. *Powder Technol.*, **247**, 1–7.
[102] Langrish, T.A.G. (2007) New engineered particles from spray dryers: Research needs in spray drying. *Drying Technol.*, **25**(6), 971–983.
[103] Kuriakose, R. and Anandharamakrishnan, C. (2010) Computational fluid dynamics (CFD) applications in spray drying of food products. *Trends Food Sci. Technol.*, **21**(8), 383–398.
[104] Petrides, D.P., Koulouris, A. and Lagonikos, P.T. (2002) The role of process simulation in pharmaceutical process development and product commercialization. *Pharm. Eng.* [Internet], **22**(1), [1–8 pp.].
[105] Zhang, J., Wu, L., Chan, H.-K. and Watanabe, W. (2011) Formation, characterization, and fate of inhaled drug nanoparticles. *Adv. Drug Deliv. Rev.*, **63**(6), 441–455.

[106] Chan, H.-K. and Kwok, P.C.L. (2011) Production methods for nanodrug particles using the bottom-up approach. *Adv. Drug Deliv. Rev.*, **63**(6), 406–416.
[107] Li, X., Anton, N., Arpagaus, C. *et al*. (2010) Nanoparticles by spray drying using innovative new technology: The büchi nano spray dryer B-90. *J. Control. Release*, **147**(2), 304–310.
[108] Lee, S.H., Heng, D., Ng, W.K. et al. (2011) Nano spray drying: A novel method for preparing protein nanoparticles for protein therapy. *Int. J. Pharm.*, **403**(1–2), 192–200.
[109] Heng, D., Lee, S.H., Ng, W.K. and Tan, R.B. (2011) The Nano Spray Dryer B-90. *Expert Opin. Drug Deliv.*, **8**(7), 965–972.
[110] Schmid, K., Arpagaus, C. and Friess, W. (2011) Evaluation of the Nano Spray Dryer B-90 for pharmaceutical applications. *Pharm. Dev. Technol.*, **16**(4), 287–294.
[111] Bürki, K., Jeon, I., Arpagaus, C. and Betz, G. (2011) New insights into respirable protein powder preparation using a Nano Spray Dryer. *Int. J. Pharm.*, **408**(1–2), 248–256.
[112] Martena, V., Censi, R., Hoti, E. *et al*. (2012) A new nanospray drying method for the preparation of nicergoline pure nanoparticles. *J. Nanopart. Res.*, **14**(6), 1–10.
[113] Brinkmann-Trettenes, U. and Bauer-Brandl, A. (2014) Solid phospholipid nano-particles: Investigations into formulation and dissolution properties of griseofulvin. *Int. J. Pharm.*, **467**(1–2), 42–47.
[114] Harsha, S., Al-Khars, M., Al-Hassan, M. *et al.* (2014) Pharmacokinetics and tissue distribution of spray-dried carboplatin microspheres: Lung targeting via intravenous route. *Arch. Pharm. Res.*, **37**(3), 352–360.
[115] Lee, S.H., Teo, J., Heng, D. *et al*. (2013) Synergistic combination dry powders for inhaled antimicrobial therapy: Formulation, characterization and in vitro evaluation. *Eur. J. Pharm. Biopharm.*, **83**(2), 275–284.
[116] Ramtoola, Z. (inventor) (2008) Method of producing microcapsules. Ireland Patent. WO/2008/056344. WIPO.
[117] Kašpar, O., Jakubec, M. and Štěpánek, F. (2013) Characterization of spray dried chitosan–TPP microparticles formed by two- and three-fluid nozzles. *Powder Technol.*, **240**, 31–40.
[118] Pabari, R.M., Sunderland, T. and Ramtoola, Z. (2012) Investigation of a novel 3-fluid nozzle spray drying technology for the engineering of multifunctional layered microparticles. *Expert Opin. Drug Deliv.*, **9**(12), 1463–1474.
[119] Wan, F., Maltesen, M., Andersen, S. *et al*. (2014) One-step production of protein-loaded PLGA microparticles via spray drying using 3-fluid nozzle. *Pharm. Res.*, **31**(8), 1967–1977.
[120] Wan, F., Maltesen, M., Andersen, S. *et al.* (2014) Modulating protein release profiles by incorporating hyaluronic acid into PLGA microparticles via a spray dryer equipped with a 3-fluid nozzle. *Pharm. Res.*, **31**(11), 2940–2951.
[121] Kondo, K., Niwa, T. and Danjo, K. (2014) Preparation of sustained-release coated particles by novel microencapsulation method using three-fluid nozzle spray drying technique. *Eur. J. Pharm. Sci.*, **51**, 11–19.
[122] Wang, C. and Hickey, A.J. (2010) Isoxyl aerosols for tuberculosis treatment: Preparation and characterization of particles. *AAPS PharmSciTech.*, **11**(2), 538–549.
[123] Legako, J. and Dunford, N.T. (2010) Effect of spray nozzle design on fish oil–whey protein microcapsule properties. *J. Food Sci.*, **75**(6), E394–E400.
[124] Son, Y.-J. and McConville, J.T. (2012) Preparation of sustained release rifampicin microparticles for inhalation. *J. Pharm. Pharmacol.*, **64**(9), 1291–1302.
[125] Jiang, H., Zhang, M., McKnight, S. and Adhikari, B. (2013) Microencapsulation of α-amylase by carrying out complex coacervation and drying in a single step using a novel three-fluid nozzle spray drying. *Drying Technol.*, **31**(16), 1901–1910.
[126] Mizoe, T., Ozeki, T. and Okada, H. (2007) Preparation of drug nanoparticle-containing microparticles using a 4-fluid nozzle spray drier for oral, pulmonary, and injection dosage forms. *J. Control. Release*, **122**(1), 10–15.
[127] Mizoe, T., Ozeki, T. and Okada, H. (2008) Application of a four-fluid nozzle spray drier to prepare inhalable rifampicin-containing mannitol microparticles. *AAPS PharmSciTech.*, **9**(3), 755–761.
[128] Ohashi, K., Kabasawa, T., Ozeki, T. and Okada, H. (2009) One-step preparation of rifampicin/poly(lactic-co-glycolic acid) nanoparticle-containing mannitol microspheres using a four-fluid nozzle spray drier for inhalation therapy of tuberculosis. *J. Control. Release*, **135**(1), 19–24.

[129] Chen, R., Tagawa, M., Hoshi, N. *et al.* (2004) Improved dissolution of an insoluble drug using a 4-fluid nozzle spray-drying technique. *Chem. Pharm. Bull.*, **52**(9), 1066–1070.
[130] Ozeki, T., Beppu, S., Mizoe, T. *et al.* (2005) Preparation of two-drug composite microparticles to improve the dissolution of insoluble drug in water for use with a 4-fluid nozzle spray drier. *J. Control. Release*, **107**(3), 387–394.
[131] Mizoe, T., Beppu, S., Ozeki, T. and Okada, H. (2007) One-step preparation of drug-containing microparticles to enhance the dissolution and absorption of poorly water-soluble drugs using a 4-fluid nozzle spray drier. *J. Control. Release*, **120**(3), 205–210.
[132] Chen, R., Okamoto, H. and Danjo, K. (2008) Preparation of functional composite particles of salbutamol sulfate using a 4-fluid nozzle spray-drying technique. *Chem. Pharm. Bull.*, **56**(3), 254–259.
[133] Ozeki, T., Kano, Y., Takahashi, N. *et al.* (2012) Improved bioavailability of a water-insoluble drug by inhalation of drug-containing maltosyl-β-cyclodextrin microspheres using a four-fluid nozzle spray drier. *AAPS PharmSciTech.*, **13**(4), 1130–1137.
[134] Ozeki, T., Akiyama, Y., Takahashi, N. *et al*. (2012) Development of a novel and customizable two-solution mixing type spray nozzle for one-step preparation of nanoparticle-containing microparticles. *Biol. Pharm. Bull.*, **35**(11), 1926–1931.
[135] Nishino, Y., Kubota, A., Kanazawa, T. *et al.* (2012) Improved intestinal absorption of a poorly water-soluble oral drug using mannitol microparticles containing a nanosolid drug dispersion. *J. Pharm. Sci.*, **101**(11), 4191–4200.
[136] Coowanitwong, I., Arya, V., Kulvanich, P. and Hochhaus, G. (2008) Slow release formulations of inhaled rifampin. *AAPS J.*, **10**(2), 342–348.
[137] O'Hara, P. and Hickey, A.J. (2000) Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: Manufacture and characterization. *Pharm. Res.*, **17**(8), 955–961.
[138] Pandey, R. and Khuller, G.K. (2004) Polymer based drug delivery systems for mycobacterial infections. *Curr. Drug Deliv.*, **1**(3), 195–201.
[139] Bain, D.F., Munday, D.L. and Smith, A. (1999) Solvent influence on spray-dried biodegradable microspheres. *J. Microencapsul.*, **16**(4), 453–474.
[140] Sung, J., Padilla, D., Garcia-Contreras, L. *et al.* (2009) Formulation and pharmacokinetics of self-assembled rifampicin nanoparticle systems for pulmonary delivery. *Pharm. Res.*, **26**(8), 1847–1855.
[141] Son, Y.-J. and McConville, J.T. (2011) A new respirable form of rifampicin. *Eur. J. Pharm. Biopharm.*, **78**(3), 366–376.
[142] Chan, J.G.Y., Chan, H.-K., Prestidge, C.A. *et al*. (2013) A novel dry powder inhalable formulation incorporating three first-line anti-tubercular antibiotics. *Eur. J. Pharm. Biopharm.*, **83**(2), 285–292.
[143] Chan, J.G.Y., Duke, C.C., Ong, H.X. *et al.* (2014) A novel inhalable form of rifapentine. *J. Pharm. Sci.*, **103**(5), 1411–1421.
[144] Chan, J.G.Y., Tyne, A., Pang, A. *et al.* (2014) A rifapentine-containing inhaled triple antibiotic formulation for rapid treatment of tubercular infection. *Pharm. Res.*, **31**(5), 1239–1253.
[145] Darbandi, M., Gilani, K., Najafabadi, A.R. *et al*. (2008) The effect of spray drying solvent on in vitro deposition profiles and pulmonary absorption of rifampicin microparticles. *J. Drug Deliv. Sci. Technol.*, **18**(3), 203–208.
[146] Rojanarat, W., Changsan, N., Tawithong, E. *et al.* (2011) Isoniazid proliposome powders for inhalation—preparation, characterization and cell culture studies. *Int. J. Mol. Sci.*, **12**(7), 4414–4434.
[147] Rojanarat, W., Nakpheng, T., Thawithong, E. *et al*. (2012) Inhaled pyrazinamide proliposome for targeting alveolar macrophages. *Drug Deliv.*, **19**(7), 334–345.
[148] Pham, D.-D., Fattal, E., Ghermani, N. *et al.* (2013) Formulation of pyrazinamide-loaded large porous particles for the pulmonary route: Avoiding crystal growth using excipients. *Int. J. Pharm.*, **454**(2), 668–677.
[149] Rojanarat, W., Nakpheng, T., Thawithong, E. *et al.* (2012) Levofloxacin-proliposomes: Opportunities for use in lung tuberculosis. *Pharmaceutics*, **4**(3), 385–412.
[150] Hwang, S.M., Kim, D.D., Chung, S.J. and Shim, C.K. (2008) Delivery of ofloxacin to the lung and alveolar macrophages via hyaluronan microspheres for the treatment of tuberculosis. *J. Control. Release*, **129**(2), 100–106.

[151] Muttil, P., Kaur, J., Kumar, K. *et al.* (2007) Inhalable microparticles containing large payload of anti-tuberculosis drugs. *Eur. J. Pharm. Sci.*, **32**(2), 140–150.
[152] Booysen, L.L.I.J., Kalombo, L., Brooks, E. *et al.* (2013) In vivo/in vitro pharmacokinetic and pharmacodynamic study of spray-dried poly-(dl-lactic-co-glycolic) acid nanoparticles encapsulating rifampicin and isoniazid. *Int. J. Pharm.*, **444**(1–2), 10–17.
[153] Verma, R.K., Singh, A.K., Mohan, M. *et al.* (2012) Inhalable microparticles containing nitric oxide donors: Saying no to intracellular mycobacterium tuberculosis. *Mol. Pharm.*, **9**(11), 3183–3189.
[154] Sawatdee, S., Worakul, N. and Srichana, T. (2006) Preparation of isoniazid as dry powder formulations for inhalation by physical mixing and spray drying. *Malaysian J. Pharm. Sci.*, **4**(1), 43–63.
[155] Garcia-Contreras, L., Fiegel, J., Telko, M.J. *et al.* (2007) Inhaled large porous particles of capreomycin for treatment of tuberculosis in a guinea pig model. *Antimicrob. Agents Chemother.*, **51**(8), 2830–2836.
[156] Fiegel, J., Garcia-Contreras, L., Thomas, M. *et al*. (2008) Preparation and in vivo evaluation of a dry powder for inhalation of capreomycin. *Pharm. Res.*, **25**(4), 805–811.
[157] Schoubben, A., Blasi, P., Giovagnoli, S. *et al.* (2010) Simple and scalable method for peptide inhalable powder production. *Eur. J. Pharm. Sci.*, **39**(1–3), 53–58.
[158] Tsapis, N., Bennett, D., O'Driscoll, K. *et al.* (2003) Direct lung delivery of para-aminosalicylic acid by aerosol particles. *Tuberculosis*, **83**(6), 379–385.
[159] Durham, P.G., German, N., Zhang, Y. *et al.* (eds). (2013) *Spray dried pyrazinoate salts for tuberculosis therapy*. AAPS Annual Meeting, San Antonio, Texas, 2013.
[160] Sung, J.C., Garcia-Contreras, L., VerBerkmoes, J.L. *et al.* (2009) Dry powder nitroimidazopyran antibiotic PA-824 aerosol for inhalation. *Antimicrob. Agents Chemother.*, **53**(4), 1338–1343.
[161] Wong, Y.-L., Sampson, S., Germishuizen, W.A. *et al.* (2007) Drying a tuberculosis vaccine without freezing. *Proc. Natl. Acad. Sci. USA*, **104**(8), 2591–2595.
[162] Jin, T.H., Tsao, E., Goudsmit, J. *et al*. (2010) Stabilizing formulations for inhalable powders of an adenovirus 35-vectored tuberculosis (TB) vaccine (AERAS-402). *Vaccine*, **28**(27), 4369–4375.
[163] Lu, D., Garcia-Contreras, L., Xu, D. *et al.* (2007) Poly (lactide-co-glycolide) microspheres in respirable sizes enhance an in vitro t cell response to recombinant mycobacterium tuberculosis antigen 85b. *Pharm. Res.*, **24**(10), 1834–1843.
[164] Tyne, A.S., Chan, J.G.Y., Shanahan, E.R. *et al.* (2013) TLF-2-targeted secreted proteins from mycobacterium tuberculosis are protective as powdered pulmonary vaccines. *Vaccine*, **31**(40), 4322–4329.
[165] Pandey, R. and Ahmad, Z. (2011) Nanomedicine and experimental tuberculosis: Facts, flaws, and future. *Nanomed. Nanotechnol. Biol. Med.*, **7**(3), 259–272.
[166] Suarez, S., O'Hara, P., Kazantseva, M. *et al.* (2001) Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: Screening in an infectious disease model. *Pharm. Res.*, **18**(9), 1315–1319.
[167] Sharma, R., Saxena, D., Dwivedi, A. and Misra, A. (2001) Inhalable microparticles containing drug combinations to target alveolar macrophages for treatment of pulmonary tuberculosis. *Pharm. Res.*, **18**(10), 1405–1410.
[168] Amass, W., Amass, A. and Tighe, B. (1998) A review of biodegradable polymers: Uses, current developments in the synthesis and characterization of biodegradable polyesters, blends of biodegradable polymers and recent advances in biodegradation studies. *Polym. Int.*, **47**(2), 89–144.
[169] Weers, J.G., Bell, J., Chan, H.-K. *et al*. (2010) Pulmonary formulations: What remains to be done? *J. Aerosol Med. Pulm. Drug Deliv.*, **23**(S2), S5–S23.
[170] Aristoff, P.A., Garcia, G.A., Kirchhoff, P.D. and Hollis Showalter, H.D. (2010) Rifamycins – obstacles and opportunities. *Tuberculosis*, **90**(2), 94–118.
[171] WHO (2010) *Guidelines for Treatment of Tuberculosis*, 4th edn, World Health Organization, 2010 [25/1/2015]. Available from: http://www.who.int/tb/publications/2010/9789241547833/en/. (Accessed 20th March 2016.)

[172] Yoshida, A., Matumoto, M., Hshizume, H. *et al.* (2006) Selective delivery of rifampicin incorporated into poly(dl-lactic-co-glycolic) acid microspheres after phagocytotic uptake by alveolar macrophages, and the killing effect against intracellular mycobacterium bovis calmette–guérin. *Microbes Infect.*, **8**(9–10), 2484–2491.
[173] Suarez, S., O'Hara, P., Kazantseva, M. *et al.* (2001) Airways delivery of rifampicin microparticles for the treatment of tuberculosis. *J. Antimicrob. Chemother.*, **48**(3), 431–434.
[174] Abubakar, I., Zignol, M., Falzon, D. *et al*. (2013) Drug-resistant tuberculosis: Time for visionary political leadership. *Lancet Infect. Dis.*, **13**(6), 529–539.
[175] Mariappan, T.T., Geetha, T., Pandey, R. *et al*. (2004) Interference of isonicotinyl hydrazone in the microbiological analysis of rifampicin from anti-tuberculosis FDC products containing isoniazid. *J. Pharm. Biomed. Anal.*, **36**(3), 643–647.
[176] Shishoo, C.J., Shah, S.A., Rathod, I.S. *et al.* (2001) Impaired bioavailability of rifampicin in presence of isoniazid from fixed dose combination (FDC) formulation. *Int. J. Pharm.*, **228**(1–2), 53–67.
[177] Miller, B.H., Fratti, R.A., Poschet, J.F. *et al.* (2004) Mycobacteria inhibit nitric oxide synthase recruitment to phagosomes during macrophage infection. *Infect. Immun.*, **72**(5), 2872–2878.
[178] Darbandi, M.A., Rouholamini, N.A., Gilani, K. and Tajerzadeh, H. (2008) The effect of vehicles on spray drying of rifampicin inhalable microparticles: In vitro and in vivo evaluation. *DARU - J. Fac. Pharm., Tehran Univ. Med. Sci.*, **16**(3), 128–135.
[179] Garcia-Contreras, L., Muttil, P., Fallon, J.K. *et al*. (2012) Pharmacokinetics of sequential doses of capreomycin powder for inhalation in guinea pigs. *Antimicrob. Agents Chemother.*, **56**(5), 2612–2618.
[180] Dharmadhikari, A.S., Kabadi, M., Gerety, B. et al. (2013) Phase I, single-dose, dose-escalating study of inhaled dry powder capreomycin: A new approach to therapy of drug-resistant tuberculosis. *Antimicrob. Agents Chemother.*, **57**(6), 2613–2619.
[181] Zhang, Y. and Mitchison, D. (2003) The curious characteristics of pyrazinamide: A review. *Int. J. Tuberc. Lung Dis.*, **7**(1), 6–21.
[182] Ginsberg, A.M., Laurenzi, M.W., Rouse, D.J. *et al.* (2009) Safety, tolerability, and pharmacokinetics of PA-824 in healthy subjects. *Antimicrob. Agents Chemother.*, **53**(9), 3720–3725.
[183] Diacon, A.H., Dawson, R., du Bois, J. *et al.* (2012) Phase II dose-ranging trial of the early bactericidal activity of PA-824. *Antimicrob. Agents Chemother.*, **56**(6), 3027–3031.
[184] Garcia-Contreras, L., Sung, J.C., Muttil, P. *et al*. (2010) Dry powder pa-824 aerosols for treatment of tuberculosis in guinea pigs. *Antimicrob. Agents Chemother.*, **54**(4), 1436–1442.
[185] Garcia-Contreras, L., Wong, Y.-L., Muttil, P. *et al.* (2008) Immunization by a bacterial aerosol. *Proc. Natl. Acad. Sci.*, **105**(12), 4656–4660.
[186] Hokey, D.A. and Misra, A. (2011) Aerosol vaccines for tuberculosis: A fine line between protection and pathology. *Tuberculosis*, **91**(1), 82–85.
[187] Yadav, A.B., Singh, A.K., Verma, R.K. *e*t al. (2011) The devil's advocacy: When and why inhaled therapies for tuberculosis may not work. *Tuberculosis*, **91**(1), 65–66.
[188] Belisle, J.T., Vissa, V.D., Sievert, T. *et al.* (1997) Role of the major antigen of mycobacterium tuberculosis in cell wall biogenesis. *Science*, **276**(5317), 1420–1422.
[189] Ulmer, J.B., Liu, M.A., Montgomery, D.L. *et al*. (1997) Expression and immunogenicity of mycobacterium tuberculosis antigen 85 by DNA vaccination. *Vaccine*, **15**(8), 792–794.
[190] Lozes, E., Huygen, K., Content, J. *et al.* (1997) Immunogenicity and efficacy of a tuberculosis DNA vaccine encoding the components of the secreted antigen 85 complex. *Vaccine*, **15**(8), 830–833.
[191] Horwitz, M.A., Harth, G., Dillon, B.J. and Masleša-Galić, S. (2000) Recombinant Bacillus Calmette–Guérin (BCG) vaccines expressing the mycobacterium tuberculosis 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. *Proc. Natl. Acad. Sci.*, **97**(25), 13853–13858.
[192] Miki, K., Nagata, T., Tanaka, T. *et al.* (2004) Induction of protective cellular immunity against mycobacterium tuberculosis by recombinant attenuated self-destructing listeria monocytogenes strains harboring eukaryotic expression plasmids for antigen 85 complex and MPB/MPT51. *Infect. Immun.*, **72**(4), 2014–2021.

[193] Gler, M.T., Skripconoka, V., Sanchez-Garavito, E. *et al*. (2012) Delamanid for multidrug-resistant pulmonary tuberculosis. *New Engl. J. Med.*, **366**(23), 2151–2160.
[194] Singh, R., Manjunatha, U., Boshoff, H.I.M. *et al.* (2008) PA-824 kills nonreplicating mycobacterium tuberculosis by intracellular NO release. *Science*, **322**(5906), 1392–1395.
[195] Nikonenko, B.V., Protopopova, M., Samala, R. *et al.* (2007) Drug therapy of experimental tuberculosis (TB): Improved outcome by combining SQ109, a new diamine antibiotic, with existing TB drugs. *Antimicrob. Agents Chemother.*, **51**(4), 1563–1565.
[196] Lanoix, J.P. and Nuermberger, E. (2013) Sutezolid oxazolidinone antibacterial treatment of tuberculosis. *Drugs Future*, **38**(6), 387–394.
[197] Salomon, J.J., Galeron, P., Schulte, N. *et al*. (2013) Biopharmaceutical in vitro characterization of CPZEN-45, a drug candidate for inhalation therapy of tuberculosis. *Ther. Deliv.*, **4**(8), 915–923.
[198] Hoshino, K., Inoue, K., Murakami, Y. *et al.* (2008) In vitro and in vivo antibacterial activities of DC-159a, a new fluoroquinolone. *Antimicrob. Agents Chemother.*, **52**(1), 65–76.
[199] Nikonenko, B.V., Reddy, V.M., Protopopova, M. *et al.* (2009) Activity of SQ641, a capuramycin analog, in a murine model of tuberculosis. *Antimicrob. Agents Chemother.*, **53**(7), 3138–3139.
[200] Balasubramanian, V., Solapure, S., Iyer, H. *et al.* (2014) Bactericidal activity and mechanism of action of AZD-5847, a novel oxazolidinone for treatment of tuberculosis. *Antimicrob. Agents Chemother.*, **58**(1), 495–502.
[201] Rowland, R. and McShane, H. (2011) Tuberculosis vaccines in clinical trials. *Expert Rev. Vaccines*, **10**(5), 645–658.
[202] Hickey, A.J., Misra, A. and Fourie, P.B. (2013) Dry powder antibiotic aerosol product development: Inhaled therapy for tuberculosis. *J. Pharm. Sci.*, **102**(11), 3900–3907.
[203] Ramón-García, S., Ng, C., Anderson, H. *et al.* (2011) Synergistic drug combinations for tuberculosis therapy identified by a novel high-throughput screen. *Antimicrob. Agents Chemother.*, **55**(8), 3861–3869.

10

Formulation Strategies for Antitubercular Drugs by Inhalation

Francesca Buttini[a,b] *and Gaia Colombo*[c]

[a] *Department of Pharmacy, University of Parma, Parma, Italy*
[b] *Institute of Pharmaceutical Science, King's College London, London, UK*
[c] *Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy*

10.1 Introduction

Pulmonary tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*, most commonly affecting the lungs. It is transmitted from one person to another via contaminated droplets nebulized in air when people with the active infection cough or sneeze.

Often, *Mycobacterium tuberculosis* infection is asymptomatic, since the person's immune system acts to "wall off" the bacteria. These germs usually attack the lungs, but they can also be found in other parts of the body, such as kidneys, brain or spine.

Tuberculosis is treated with antibacterial drugs following a therapy schedule lasting six months. Drugs are mainly given by the oral route. Thus, TB is treated by a combination of several drugs. It is very important that people with TB strictly adhere to the medicine-administration scheme, taking the drugs as prescribed. In some situations, staff of the local health department meet regularly with the patients to watch them taking medications. This is called "directly observed therapy" and helps the patient to complete the treatment in the

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

proper period of time. Ceasing taking the drugs too soon or taking them incorrectly may select bacteria strains that are resistant to the drugs. If tuberculosis is resistant to drugs, it is tougher and more expensive to treat.

One drug alone is unlikely to be active against all populations of *M. tuberculosis* complex bacilli (i.e., actively multiplying bacilli, slowly or sporadically multiplying bacilli and dormant bacilli). Two-drug regimens might, in theory, be active against all populations, and three-drug combinations might show even higher activity. There are 10 drugs currently approved by the U.S. Food and Drug Administration (FDA) for treating TB [1]). Among the approved drugs, the first-line anti-TB agents that form the core treatment regimen include four orally associated drug substances, i.e., rifampicin (R), isoniazid (H), pyrazinamide (Z) and ethambutol (E). In the EU there is a request for optimizing the therapeutic management of TB disease, particularly in children, focusing on the appropriate dosing. The harmonization of doses passes through the development of fixed dose combinations (FDCs) by pharmaceutical companies. FDC tablets can facilitate the adherence, helping the patient follow the medical prescription by taking the medicines at the right time and in the correct combination and number. FDCs simplify drug prescription and supply, and contribute to limiting the risks of drug-resistant tuberculosis resulting from inappropriate drug selection, mono-therapy and poor convenience and compliance.

Drug-resistant TB is the man-made result of interrupted, erratic, or inadequate TB therapy, and its expansion is undermining efforts to control the global TB epidemic. Once a drug-resistant strain has developed, it can be transmitted directly to other individuals just like drug-susceptible TB bacteria.

The therapy of multidrug-resistant tuberculosis (MDR-TB) is carried on with the so-called second-line drugs (Table 10.1). These drugs are administered when first-line drugs fail. Treatment for MDR-TB is continued for 2 years or longer and involves daily injections for six months. A daily intramuscular and/or intravenous injection is a painful and difficult-to-perform procedure among many patients. An exclusively oral regimen would be easier to manage. Several studies have recently been started in order to introduce inhalation medicines into this scenario that enable to complement the oral or parenteral administration, eventually aiming to increase the effectiveness against the resistant strains. However, no product for inhalation is yet available to treat TB.

Thus, the advent of MDR-TB has raised great interest in the administration of drugs by inhalation, utilizing antibiotics or other drugs otherwise administered by injection. Among drugs proposed for this administration route, aminoglycoside antibiotics such as kanamycin and its derivative amikacin are possible candidates.

10.2 Lung Delivery of TB Drugs

Special attention has to be dedicated to the quality of the dosage forms for TB therapy, in particular concerning the bioavailability. In many cases, blood levels of the oral TB drugs are inadequate because of poor medicine quality rather than poor absorption. Therefore, formulation approaches have to assure high-quality products for therapy, in particular when proposing a new administration route.

Since the lung is the primary entrance for the mycobacterium, the interest in the management of TB by delivering drugs through the same gateway is well justified. In recent years

Table 10.1 *List of anti-TB drugs and doses for adults (IV: intravenous; IM: intramuscular; XMDR: extensively multidrug-resistant tuberculosis) (adapted from ref. [1])*

	Administration route	Recommended daily dose
1. First-line drugs		
1.1 Oral agents		
Isoniazid	Oral	300 mg
Rifampicin	Oral	600 mg
Pyrazinamide	Oral	1–2 g
Ethambutol	Oral	0.8–1.6 g
Rifabutin	Oral	300 mg
2. Second-line drugs		
2.1 Injectable agents		
Amikacin/Kanamicin	IV-IM	0.5–1 g
Capreomycin	IV-IM	1 g
Streptomycin	IV-IM	1 g
2.2 Fluoroquinolones		
Levofloxacin	Oral/IV IM	0.5–1 g
Moxifloxacin	Oral/IV IM	400 mg
2.3 Oral bacteriostatic second-line agents		
p-Aminosalicylic acid	Oral	8–12 g
Cycloserine	Oral	500–750 mg
Terizidone[a]	—	—
2.4 Agents with unclear role in the treatment of XMDR		
Clofazimine[a]	—	—
Linezolid[a]	—	—
Imipenem/Cilastatin[a]	—	—
Amoxicillin/Clavulanate[a]	—	—

[a] No data are available at the moment for these five compounds/mixtures that might become available for clinical evaluation in the future.

there has been growing interest in formulating anti-TB drugs directly targeting the lung. Moreover, the lung macrophages pick up the mycobacteria and the drug microparticles at the same time, determining a direct contact between drug and TB bacilli.

It must not be considered that pulmonary administration will determine the effective plasma level of the drug for antimicrobial activity. The absorptive capacity of the lung is undoubtedly high, but the amount of drug deliverable is limited. Thus, the administration by inhalation would complement, integrate and strengthen the systemic therapy by creating a localized drug concentration in contact with the microorganism. Moreover, when it comes to the formulation, the persistence of local drug levels could be attained by designing a product intended for prolonging drug release. Some TB second-line drugs administered by the parenteral route are the logical candidates for inhalation aiming to reinforce the standard therapy, certainly not to substitute it.

The first constraint in formulating antibiotics for lung delivery is the high amount of drug to deliver. There are anti-TB drugs that are given orally or by injection at dosages in the order of hundreds of milligrams. It could be that direct delivery to the lung allows for a reduction in the dose compared with oral or injectable routes, but this possibility has yet to

be demonstrated. The second constraint to face is the need in TB treatment for drug combinations, which will make the formulation decisions more complex for the dosage form preparation.

The typical inhalation drug product for aerosol delivery at high doses is a drug solution or suspension for nebulization. The patient tidally inhales the aerosol produced by the nebulizer through the mouthpiece. Solutions or suspensions are not a difficult task for the formulator, but in TB treatment the adherence to this dosage form, requiring long administration time and suitable equipment, is not straightforward outside a care environment. These considerations push towards inhalation products capable of reproducing the release specifications with minimum use of time, resources and cost. In general, these are crucial elements in the development of inhalation products. Therefore, even if the environmental and socio-economical conditions allow for nebulizer use, the preference goes to formulations that speed up and simplify the administration, as should be possible with dry-powder inhalers (DPIs). Nebulizers efficiently aerosolize solutions of antibiotic drugs, but it is unreasonable to propose their use in developing countries. The absence of education services makes it hard for patients to properly take their medicine and to put into practice the hygiene required to tackle the spread of infectious diseases [2]. For instance, the lack of clean water severely hampers health.

This chapter will turn its attention to the formulation of inhalation products that could be employed in several critical therapeutic and socio-economical situations with expected greater efficacy in terms of the amount of drug deposited and local drug availability.

10.3 Powders for Inhalation and Liquids for Nebulization

The complexity of engineering anti-TB drugs into respirable particles, using safe and accepted excipients and industrially scalable processes, is stressed by a number of papers in the recent literature [3]. Aerosolized solid particles need size, shape and density promoting their aerodynamic behavior; that is, transport in and sedimentation from an air stream. For clinical relevance in topical treatment of TB by inhalation, particles with adequate drug loading must reach the airways where they have to release active molecules to the target site. It is recognized that drug microparticles offer improved stability, prolonged drug action, enhanced local availability and, for poorly water-soluble substances, targeting properties toward specific cells. In addition, small nanoparticles (NPs) (<200 nm) could add further benefit to the therapy, provided that specific formulation issues (e.g., physical instability, size changes during storage, etc.) have been solved [4].

As alternatives to solutions for nebulization, dry powders give reasonable expectations of higher local bioavailability due to the large number of drug molecules per lung mass that comes from drug particles taken in by aerosol as compared with systemic administration.

Recently, two antibiotic drugs, already given by nebulization, have been registered as DPIs. Tobramycin and colistimethate, used for treating *Pseudomonas aeruginosa* (PA) infections, have been reformulated in dry powders for inhalation. They are not used in treating TB, but the formulation decisions adopted are instructive for the proposition of other drugs. The proposal of a DPI product for these drug substances aimed to simplify their administration compared with nebulization. In fact, in PA infections administration is performed twice daily in cycles of 28 days. Tobramycin was registered as TOBI™

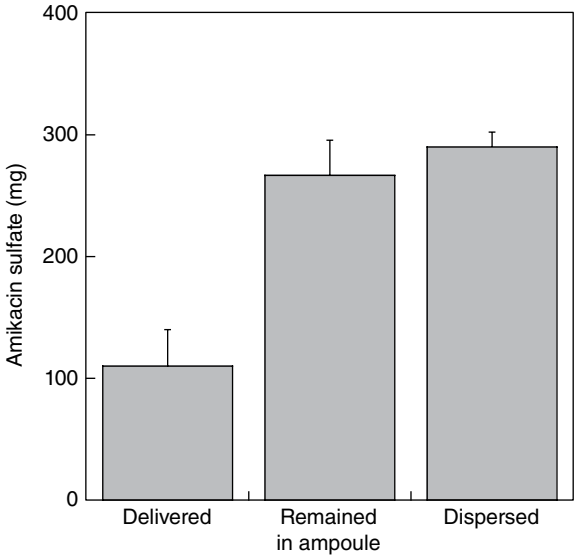


Figure 10.1 *Distribution of amikacin solution for injection after the nebulization of 667.5 mg of amikacin sulfate in 3 ml of solution (corresponding to a labeled dose of 500 mg of amikacin free base) using a Pari LC Plus nebulizer*

Podhaler™ to replace the 300 mg drug dose in 5 ml of nebulizer solution with an inhaled powder dose of 112 mg. This dose was bioequivalent to that given in the nebulizer solution [5]. Similarly, the nebulization of 160 mg of colistimethate as a solution for injection was determined as bioequivalent to 125 mg of drug powder for inhalation [6].

In formulating drugs for TB treatment by inhalation, the first aspect to solve is the dose definition. For instance, the drug dose to deposit into the lung as powder could be determined based on a comparison with the solution for use in nebulization. In some cases, physicians prescribe marketed injectable doses, provided that the tolerability and safety of pulmonary administration have been assessed. For example, amikacin sulfate solution for injection, 667.5 mg in 2 ml, has been effectively given off-label by inhalation after dilution with 1 ml of saline against PA infections (http://torontoadultcf.com/medications/inhaled-amikacin). Figure 10.1 shows the result of an *in vitro* test showing that at the end of the nebulization time about 40% of the amikacin dose remained in the ampoule, 43.5% was dispersed (lost) in the environment and only 16.5% of the loaded dose (109.9 mg) was delivered as aerosol suitable for lung deposition.

An amikacin dry powder for inhalation was tested *in vitro* in order to find the *in vitro* lung deposition similar to the amount of antibiotic deposited by nebulization. The aerodynamic assessment of amikacin solution for injection (667.5 mg of amikacin sulfate in 3 ml of solution) nebulized with one of the best performing nebulizers commercially available (Pari Turbo Boy equipped with a Pari LC Plus ampoule; PARI GmbH, Starnberg, Germany) is presented in Table 10.2. The fine-particle dose (FPD), i.e., the respirable mass of drug, was about 62 mg of amikacin sulfate. Thus, an amount of amikacin powder formulation corresponding to 125 mg of amikacin sulfate was introduced into a hard capsule and

Table 10.2 *Aerodynamic parameters measured for amikacin solution for injection nebulized using Pari LC Plus apparatus and amikacin spray-dried powder in RS01 device*

	AMK sulfate loaded dose (mg)	Emitted dose aerosol output (mg)	FPD (mg)	FPF < 5 μm (%)
Amikacin powder	125.0	114.6 ± 6.4	63.8 ± 0.6	65.8 ± 2.3
Amikacin injection	667.5	109.9 ± 29.8	61.9[a] ± 0.1	56.3 ± 0.1

[a] Calculated from the aerosol output and the respirable fraction (FPF).

aerosolized using the RS01 DPI (Plastiape, Osnago, LC, Italy). The result obtained shows that the FPD (63.8 mg of amikacin sulfate), that is the amount of aerosol particles deposited on the impactor stages considered as respirable particle-size limit, was similar to the amount measured with the nebulized amikacin solution.

Therefore, the use of dry powder could sensibly reduce the amount of drug to inhale, at least *in vitro*, but this amount still remains notably high for dry-powder inhalation. Having established that for certain antibacterial drugs the possibility exists to identify the inhalation dose by comparing the nebulized drug and inhaled amounts, in TB therapy the combination of different drugs is also needed in order to achieve effectiveness of the therapy. Combining several drugs in a nebulizer solution is a matter of chemical compatibility between the molecules. Here, issues are not as difficult as they can be with blending of different powder drugs to obtain a homogeneous mixture, in particular if these drugs are micronized. In addition, it has to be underlined that dosages are modified during the treatment period, which means that the formulation must have a certain level of flexibility in order to enable the dose to change over time. The strategies for facing these issues will be described in the next section.

10.4 Antibacterial Powders for Inhalation: Manufacturing of Respirable Microparticles

The dominant aspect for the formulation of a DPI is the respirability of the product. Aerosolization properties, including emission, dispersion and deposition profile, are governed by both the formulation properties and device characteristics.

The design of anti-TB drug microparticles could require the use of excipients to control the adhesion forces between particles and to construct an effective aerodynamic powder. Consequently, in the case of significant amounts of added excipients, the obtained microparticles have limited drug payload. Then, a considerable drug mass (hundreds of milligrams per dose) has to be loaded and delivered to the lung for supporting local efficacy. As a matter of fact, the ideal formulation should contain the lowest amount of adjuvants in order to avoid charging the lungs with foreign material.

In addition, the advantages of a powder for inhalation in comparison with a nebulized liquid formulation include other aspects, such as light weight and portability, which enable the dose to be taken quickly and easily in everyday life situations.

We illustrated earlier that, compared with solutions for nebulization, the use of dry-powder formulations is expected to decrease the nominal dose, since the drug loss in the

device, in the exhaled air and the environment is minimized. The benefit is evident: a respirable dose similar or even higher to that generated by nebulizing a solution for injection can be deposited into the lungs. In detail, lung deposition of the powder will create high local concentrations, likely saturation, certainly higher than those obtained with the nebulized drug solution. DPIs have the capability of dispersing a high amount of drug, such as in the case of aminoglycoside antibiotics, in a single and quick inhalation act (125 mg in 1–2 minutes) [7]. Therefore, lowering the antibiotic dose using a powder gives a major contribution to patient care, due both to the higher expected efficacy and the more convenient mode of administration deriving from the active substances' characteristics.

Particles for inhalation are very often "constructed" by using powder technologies, such as spray drying, including structural excipients to achieve inhalation-appropriate surface characteristics [8]. The development of antibiotic powder for inhalation started from the consideration that the substance in the solid state had to be protected from humidity [9]. A patented technology platform [10] claims the preparation of antibiotic microparticles whose respirability is enhanced by the presence of a small amount of sodium stearate (1% w/w) deposited on the particle surface during spray drying. Sodium stearate on the surface of amikacin, tobramycin or ciprofloxacin spray-dried particles behaves like a protective coating, making the particle surface more hydrophobic. The technology consists in dispersing the small amount of sodium stearate in the antibiotic solution to be spray dried as a hydrophobic adjunct. The surface activity of the stearate, dissolved in the solution, moves the molecules at the air/liquid interface of the sprayed droplets. Therefore, during droplet drying, the stearate molecules concentrate on the surface of the obtained particles [11]. This molecular coating protects the hygroscopic substances from the environmental humidity, keeps very high the drug content of the particle and increases the aerosolization properties. The aerodynamic spherical structures of the microparticles composing such a powder are reproduced in Figure 10.2.

The production of an antibacterial powder by spray drying using a lipophilic adjuvant, such as fatty acids in low amount, reduces the water uptake of microparticles. Tobramycin, amikacin and ciprofloxacin spray-dried powders in the presence of fatty acids were prepared in order to identify the formulation with the highest stability and respirability. The respirable fraction (FPF) of all spray-dried powders was higher than 42%. In particular, tobramycin–stearic acid spray-dried powder showed the highest FPF values (59.8%). Ciprofloxacin prepared with myristic acid {Figure 10.3(top)} had a different morphology compared with the powder without fatty acid {Figure 10.3(bottom)}. The fine-particle dose of ciprofloxacin ranged between 8–10 mg after aerosolization of 18 mg of drug coated with a saturated fatty acid (Figure 10.4).

Powders containing a fatty acid with a chain longer than 18 carbons or with the unsaturated fatty acid (oleic acid) showed low FPD values (21–32%) due to poor flowability. These products needed several actuations of the device to emit the required dose due their tendency to aggregate. A low amount of saturated fatty acid with a chain shorter than 18 carbons, added as excipient in the formulation, decreases the hygroscopic behavior of the powders and ensures the reproducibility of the emitted dose.

Amikacin spray-dried powder showed lower tendency to aggregate compared with tobramycin and ciprofloxacin. In this case, the drug spray-dried without excipient had favorable aerodynamic properties.

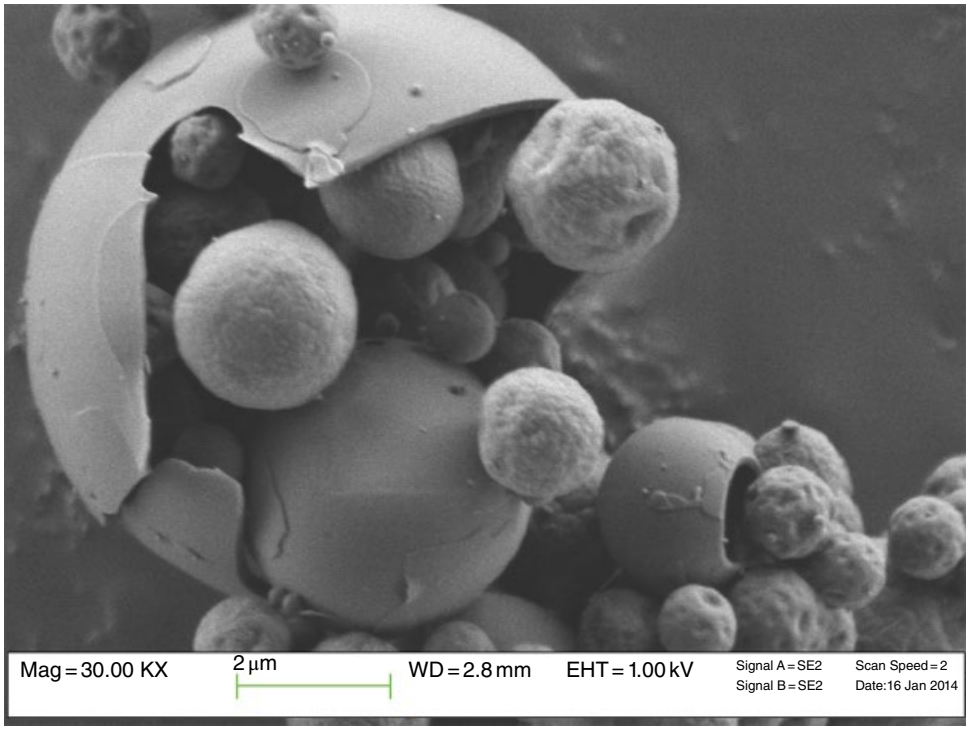


Figure 10.2 *SEM microphotograph of tobramycin powder microparticles (30 000×)*

In summary, these results demonstrated the ability of the spray drying technique to produce antibiotic dry powders using small amount of adjuvants when the molecule's physico-chemical characteristics require this addition to improve particle flowability.

In general, spray-dried antibiotic powders flow poorly because they are collected from the spray drier as particle lumps having different sizes. This makes the powder non-homogenous, anticipating negative expectations on the operations of device reservoir loading for drug product preparation (DPI). The answer to this problem is to agglomerate the micronized product by sieving to obtain microparticle clusters (Figure 10.5) [12]. The agglomeration makes the powder free-flowing and by increasing the bulk density allows for the introduction of large amounts of micronized powder in a small container, typically a capsule or a blister. Thus, the agglomeration process, performed by short sieve vibration of microparticulate powders, facilitates powder dosing into the inhaler's reservoir.

Since more than one drug is required to treat TB, the independent agglomeration of respirable microparticles of each drug in soft pellets opens up the possibility of formulating fixed dose combinations for inhalation. In fact, considering that each anti-TB drug would be individually available as soft pellets, their mixing is easy to accomplish owing to the pellets' good flow properties. Thus, the availability of soft pellets of individual anti-TB drugs allows the formulator to adjust the dose for the single patient by mixing the requested amounts of soft pellets of each drug. Therefore, the provision of microparticles agglomerated in soft clusters is a way to promote flexibility in inhalation powder delivery of combined drugs.

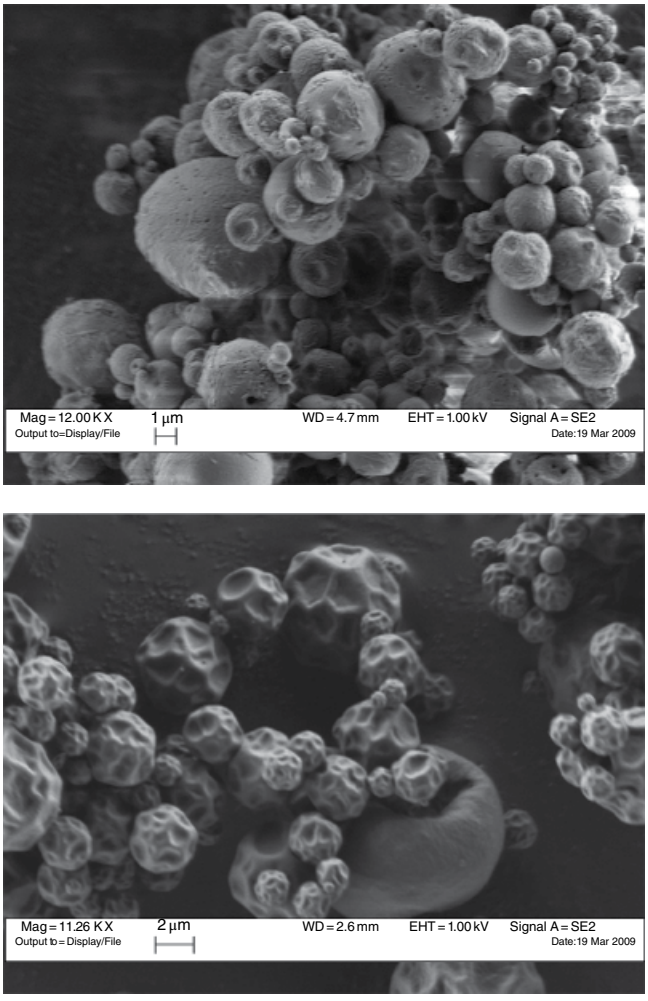


Figure 10.3 *Ciprofloxacin microparticles obtained by spray drying a solution with myristic acid (top) and without excipient (bottom)*

Alternatively to a final mixing process to combine different drugs in one single dose to administer, the construction of composite particles suggested by Traini and co-workers revealed interesting aspects for drug delivery [13]. Anti-tubercular drugs were combined into a microparticle of dry powder formulation for efficient treatment of TB patients. The micronized dry powder was produced in a single-step spray drying process without excipients and combines three first-line antibiotics for TB, as suggested by the World Health Organization (WHO): pyrazinamide, rifampicin and isoniazid in the proportions of approximately 5:2:1. Time-of-Flight Secondary Ion Mass Spectrometry analysis revealed that rifampicin remained at the particle surface, while isoniazid and pyrazinamide migrated inwards with the relatively polar solvent mixture during particle formation. The combination of three antibiotics displayed better physical stability characteristics than did powders

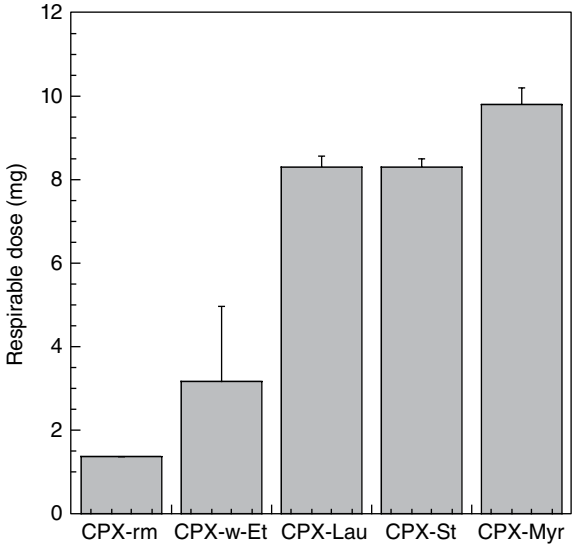


Figure 10.4 *Respirability of ciprofloxacin (CPX) spray-dried powders prepared with fatty acids (CPX-rm = raw material; CPX-w-Et = CPX spray-dried from a water:ethanol solution; CPX-Lau = CPX spray-dried from a water:ethanol solution containing lauric acid; CPX-St = CPX spray-dried from a water:ethanol solution containing stearic acid; CPX-Myr = CPX spray-dried from a water:ethanol solution containing myristic acid). The loaded dose was equal to 18 mg of CPX*

Figure 10.5 *Soft agglomerates of amikacin. Right: original spray dried powder; left: the same powder after agglomeration*

of each antibiotic spray dried individually. The presence of the more slowly dissolving rifampicin on the antibiotic particle surface was expected to reduce the overall rate of particle dissolution, thereby allowing time for some uptake by mycobacterium-infected alveolar macrophages. This strategy could offer the possibility to simultaneously administer multiple drugs to the lungs.

The capacity of specialized cells to phagocytize nanoparticles (NPs) and kill *M. tuberculosis* hidden inside these cells is related to the drug particle size. Phagocytosis is performed by macrophages and plays a role in the clearance of foreign particles having a diameter greater than 0.5 μm from the lung. It is noteworthy that macro-pinocytosis covers a broad range of particle sizes from 100 nm to 5 μm [14]. Macrophages recognize drug carrier particles as invaders, and thus "undesirable". Because *M. tuberculosis* resides and multiplies within host mononuclear phagocytes and because mononuclear phagocytes internalize particles more efficiently than do other host cells, encapsulation of drugs within NPs offers a mechanism for specifically targeting *M. tuberculosis*-infected cells. Indeed, because NPs have been shown to be taken up by macrophages of the reticuloendothelial system and to accumulate in the liver, spleen and lungs, they are ideally suited to treat *M. tuberculosis*, which infects macrophages in these organs.

NPs and colloidal formulations (like liposomes) could provide the above mentioned advantages. However, they have to be included in a suitable micron-size formulation to be efficiently administered. They can be suspended in an aqueous liquid vehicle and administered by nebulization. Arikace® liposomal product is an amikacin-containing medicinal product in the pipeline for inhalation treatment of *Pseudomonas aeruginosa* lung infection in cystic fibrosis and non-tubercular mycobacterium. This liposomal nano-suspension of amikacin (250–300 nm) is made with dipalmitoylphosphatidylcholine and cholesterol in a 2:1 weight ratio [15]. Amikacin concentration in the liposomal formulation is 70 mg/ml. The nebulization of 560 mg of amikacin, corresponding to 8 ml of liposomal dispersion, requires a long time for the administration (15–20 min), but the drug distribution and uptake in the lung is very effective. However, in this therapeutic option a nebulizer is necessary. The use of such apparatuses implies a series of time-consuming maneuvers and care for the cleaning of the ampoule and the mouthpiece.

Poly(lactic-co-glycolic) acid (PLGA) is one of the candidate polymers for drug-containing particle formulations, in particular NPs, because it is biodegradable and biocompatible [14]. Macrophage cells "eat" PLGA particles more efficiently than they do with polystyrene latex ones. It is noteworthy that phagocytosis of PLGA particles by alveolar macrophages stimulates their phagocytic activity in such a way that their uptake increases both the population of phagocytic macrophage cells and the number of particles that have been taken up by individual macrophages [16]. PLGA nanoparticles containing a high concentration of rifampicin remain membrane-enclosed in the macrophages for at least 13 days and degrade slowly. The NPs are produced with sufficient antibiotic, such that one dose given after infection is sufficient to efficiently clear the *Mycobacterium bovis* BCG after 9–12 days of treatment, as shown by estimates of the number of bacterial colonies *in vitro* [17].

From the pure or quasi-pure drug administration to the more complex delivery systems [18], mesoporous silica nanoparticle (MSNP) drug delivery systems were produced, either provided with a polyethylenimine (PEI) coating to release rifampin or with cyclodextrin-based pH-operated valves that open only at acidic pH for isoniazid release into *M. tuberculosis*-infected macrophages. The MSNP were internalized efficiently by human

macrophages, trafficked to acidified endosomes, and released high concentrations of antitubercular drugs intracellularly. These data demonstrate that MSNP provide a versatile platform that can be functionalized to optimize the loading and intracellular release of specific drugs for the treatment of tuberculosis.

Nanoparticle formulations require the design of a delivery system for administering these particles without a nebulizer for several reasons, including dispersion stability. The key concept is the disguise of the nanosized particles as microstructures delivered by inhalation. Several authors have attempted to engulf nanoparticles into microparticles [19]. Lung deposition of nanoparticles requires a temporary, but reversible, increase of their size to avoid their exhalation. To overcome these specific issues of storing and delivering nanoparticles to the lungs, a particulate form incorporating nanoparticles into micron-scale structures can be used. This type of system, defined as the Trojan Particle approach, was first proposed by Tsapis *et al*. with non-degradable particles [20]. The drug may be delivered in microparticles that also contain drug-loaded nanoparticles. Sung *et al*. formulated rifampicin (RIF) in a dry powder of 'porous nanoparticle-aggregate particle's (PNAPs) [21]. RIF was encapsulated into PLGA nanoparticles (195 nm) before being converted into PNAPs by spray drying. PNAPs had a volume median diameter of 4.2 mm and exhibited properties suitable for deposition in the respiratory tract with a FPF of 35.5–44.7%. *In vitro* release showed an initial burst of approximately 80% of RIF content, with the remaining amount available for prolonged release beyond eight hours.

10.5 Antibacterial Powders for Inhalation: Devices and Delivery Strategies

It was already mentioned that the agglomeration of the amikacin or tobramycin powders in soft pellets enables one size-0 capsule to contain a mass of powder equivalent to 110–125 mg of drug. Particle agglomerates, i.e., clusters of micronized particles, represent a strategy applied in inhalation powders already available on the market that allows a reduction of the bulk density of powders while concomitantly improving their flow rate. The agglomerates are easily destroyed by the turbulence of the air flow in the inhaler during the inhalation act, reconstituting the original particle size.

The performance of this large amount of powder loaded in one single reservoir for inhalation was demonstrated to be equivalent to the administration of discrete lower amounts, which in principle are better tolerated in one inhalation. An *in vitro* aerodynamic assessment with the Next Generation Impactor (NGI) revealed a linear relationship between the dose of antibiotic powder loaded in the capsule and the fine-particle dose emitted from capsules containing increasing amounts of powder. Figure 10.6 shows that when the labeled dose of tobramycin in the capsule was increased from 19 mg to 112 mg (i.e., from 20 to 118 mg of powder) the fine-particle mass (dose) increased linearly.

This result was confirmed using a Fast Screening Impactor (FSI) studying the deposition of spray-dried tobramycin from the device RS01 size-2 (air flow rate 90 l/min) at two drug loadings, namely 28 mg and 56 mg of tobramycin. The bar graph in Figure 10.7 also includes the deposition using the RS01 size-0 device (air flow rate 60 l/min) loaded with the equivalent of 112 mg of tobramycin. Then, despite the different flow rate and capsule size, doubling the amount of powder loaded in one capsule, the number of capsules containing the daily dose of tobramycin for inhalation halved from four size-2 capsules with

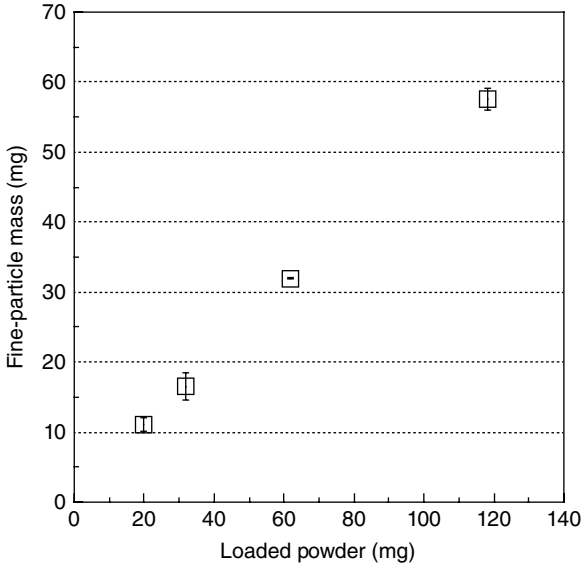


Figure 10.6 *Fine-particle mass (dose) of tobramycin versus amount of powder loaded in the capsule for tobramycin at different loadings*

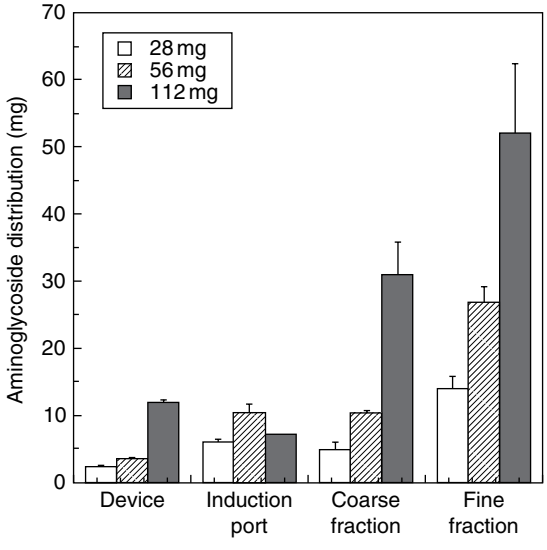


Figure 10.7 *Fast Screening Impactor deposition of spray-dried tobramycin powder (fine fraction < 5 μm)*

28 mg each to two capsules with 56 mg each and from two to one size-0 capsule with 112 mg of tobramycin.

In summary, this study illustrates a strategy for making the inhalation of large amounts of powder acceptable for the patient, i.e., the division of the high dose into several capsules

to be used in sequence with the inhaler, as already realized in the TOBI™ Podhaler™. Cumulating the total dose in one capsule and extracting it with successive inhalations is another possibility, provided that, to avoid the risk of patient choke, the amount extracted with each act is controlled by the device. The actual clinical benefit for the patient population resulting from the reduction in the number of capsules has to be studied in clinical trials, but the starting basis of less powder, fewer capsules for the same activity is a promising aspect. The increase of drug content per mg of inhalation powder is compulsory for a significant reduction of the total mass of powder entering the patient's lungs for administering the prescribed dose.

The standard device, known as RS01, manufactured by the Italian company Plastiape (Osnago, LC, Italy), works using hard capsules. Its aerosolization mechanism has been described as very efficient owing to the typical spinning of the capsule inside the device during the user's inhalation act [22]. The most classical RS01 device works with a size-3 capsule. In order to reduce the number of capsules to be used for daily administration, the tobramycin dose was cumulated in two size-2 capsules (Figure 10.7) or in one size-0 capsule, requiring the patient to extract the powder by successive inhalations. The way hard capsules are exploited as a powder reservoir in the RS01 device adds the benefit that the extraction of the powder from the reservoir is gradual because it is driven through two small holes in the capsule body and head. We tested *in vivo* the release of drug from this device and found that given a loading of 120 mg of drug in agglomerated form in a size-0 capsule, the amount of powder delivered with a short inhalation act at an inhalation peak flow of 20 l/min was around 15–20 mg of powder. This means that the complete extraction of the whole dose is obtained in 5–6 inhalations. We calculated that such a procedure requires about 2 minutes. However, it is not recommended to deliver amounts of powder higher that 10–20 mg in one shot. To do so would trigger a cough reaction in the patient with the result of dispersing and losing the inhaled amount of drug into the air. In the TOBI™ Podhaler™ the formulator identified the dose that was divided in four capsules, each one containing 28 mg of drug. In contrast, colistimethate product introduced micronized drug in one capsule with a certain level of bulk density in order to fill up the reservoir. One or two capsules to inhale instead of four would constitute a great improvement for the patient. The benefit due to less powder to be inhaled has also to be considered an improvement in safety since less excipient is deposited in the lungs.

Currently, research in device engineering focuses on proposing disposable devices, since the disease is extremely infectious and the risk of spreading microorganisms through the administration procedure is high. In addition, it must be considered that reusable devices require a cleaning procedure after use in order to maintain their performance for successive administrations. It is not difficult to presume that with a disposable medicinal product, a top benefit is provided for patient care in therapies to treat diseases like TB or lung infections. New disposable devices have been presented in order to face the requirement to administer high doses of antibacterial drugs. Two devices in particular can have a future in anti-TB preparations. They are named Cyclops [23] and Orbital [24]. It is curious to note that they both have a rotating part in their structure that helps in de-aggregating the mass of powder in order to emit respirable particles. In the case of Orbital, current interest toward the application of high doses (up to 400 mg) is determined by the fact that the device design introduced a mechanism of delivering the powder due to the inhalation effort by the user that exerts some control over the amount emitted. In general, the emission kinetics is dependent on the powder type: the more cohesive the powder, the higher the number of

required inhalations. This is not to be considered a drawback of the device since really this is a mechanism of controlling drug release for acceptance by the patient.

Cyclops is an evolution of Twincer, designed to accommodate larger amounts of powder, as it is required in antibiotic administration. It is a disposable device recommended for drug formulations in which inhaler residues can absorb moisture. Moisture absorption may hinder all subsequent inhalations with the same device as this leads to increased stickiness of the retained particles. Furthermore, a disposable inhaler eliminates the risk of bacterial resistance developing within the device and subsequent patient re-infection with drug-resistant strains of bacteria. The Cyclops has a relatively high inhaler resistance and its high dispersion efficiency and low inhaler retention enable the delivery of high dose fractions of the metered mass in the size range 1.0–5.0 µm to the respiratory tract at a flow rate of only 34 l/min. This prevents the loss of substantial drug fractions by deposition in the oropharynx. The authors say that, compared with the Podhaler™, delivering 78% of the metered mass at 72 l/min of which nearly 44% was recovered in the oropharynx, esophagus and stomach of healthy volunteers, a higher lung dose may therefore be expected [25]. Administration of that dose would even be possible in one single inhalation maneuver when the size of the blister is slightly increased, or alternatively in two inhalations when the large amount of powder appears to be inconvenient for the patient. Since the various antibiotics from this drug class may be interesting therapeutic options for a variety of diseases, the Cyclops is a valuable inhaler candidate for applications such as the treatment of TB [23, 26].

10.6 Conclusions and Perspectives

The promise to treat TB by inhalation has been shown with approaches consisting of drugs formulation and delivery strategies that appear more and more patient-centric. Drugs given by aerosol delivery systems to the lungs, in particular DPIs, reinforce the doses administered by systemic routes, attacking the problem of drug-resistant TB strains. Drug microparticles, nanoparticles and liposomes delivered to the lungs for prompt or prolonged release improved the local availability of the drug. This leads to more effective drug concentrations where *Mycobacterium tuberculosis* infection is located. Aerosol delivery to the lungs, complementing long-lasting systemic therapy, could reduce the side-effects, improve patient convenience and compliance and offer a new tool to tackle the drug-resistant strains of TB.

References

[1] Fourie, P.B and Oluwarotimi, S.N. (2015) Inhaled therapies for tuberculosis: A viable approach for spray-dried drugs delivered by handheld dry powder inhaler. *Inhalation*, **9**, 1–5.
[2] Simonsen, L., Kane, A., Lloyd, J. *et al.* (1998) Unsafe injections in the developing world and transmission of bloodborne pathogens: a review. *Bull. World Health Organ.*, **77**, 789–800.
[3] Son, Y.J. and McConville, J.T (2011) A new respirable form of rifampicin. *Eur. J. Pharm. Biopharm.*, **78**, 366–376.
[4] Ahmad Khanbeigi, R., Kumar, A., Sadouki, F. *et al*. (2012) The delivered dose: Applying particokinetics to in vitro investigations of nanoparticle internalization by macrophages. *J. Control. Release*, **162**, 259–266.
[5] Geller, D.E., Weers, J. and Heuerding, S. (2011) Development of an inhaled dry-powder formulation of tobramycin using PulmoSphere™ technology. *J. Aerosol Med. Pulm. Drug Deliv.*, **24**, 175–182.

[6] Colobreathe colistimethate sodium (rinn). Assessment Report. Procedure No. EMEA/H/C/001225. (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/001225/WC500123693.pdf)
[7] Schuster, A., Haliburn, C., Döring, G. and Goldman, M.H. (2013) Freedom Study Group, Safety, efficacy and convenience of colistimethate sodium dry powder for inhalation (Colobreathe DPI) in patients with cystic fibrosis: a randomised study. *Thorax*, **68**, 344–350.
[8] Chow, A.H., Tong, H.H., Chattopadhyay, P. and Shekunov, B.Y. (2007) Particle engineering for pulmonary drug delivery. *Pharm. Res.*, **24**, 411–437.
[9] Pilcer, G., Sebti, T. and Amighi, K. (2006) Formulation and characterization of lipid-coated tobramycin particles for dry powder inhalation. *Pharm. Res.*, **23**, 931–940.
[10] Buttini, F., Colombo, P. and Parlati, C. (2010) A drug powder for inhalation administration and a process thereof. Patent application WO2010003465 A2.
[11] Parlati, C., Colombo, C.P., Buttini, F. *et al*. (2009) Pulmonary spray dried powders of tobramycin containing sodium stearate to improve aerosolization efficiency. *Pharm. Res.*, **26**, 1084–1092.
[12] Belotti, S., Rossi, A., Colombo, P. *et al*. (2014) Spray dried amikacin powder for inhalation in cystic fibrosis patients: a quality by design approach for product construction. *Int. J. Pharm.*, **471**, 507–515.
[13] Chan, J.G., Chan, H.K., Prestidge, C.A. *et al.* (2013) A novel dry powder inhalable formulation incorporating three first-line anti-tubercular antibiotics. *Eur. J. Pharm. Biopharm.*, **83**, 285–292.
[14] Hirota, K. and Terada, H. (2012) Endocytosis of Particle Formulations by Macrophages and Its Application to Clinical Treatment, in *Molecular Regulation of Endocytosis* (ed. B. Ceresa). InTech. doi:10.5772/45820.
[15] Li, Z., Zhang, Y., Wurtz, W. *et al.* (2008) Characterization of nebulized liposomal amikacin (Arikace) as a function of droplet size. *J. Aerosol Med. Pulm. Drug Deliv.*, **21**, 245–254.
[16] Hirota, H., Hasegawa, T., Nakajima, T. *et al.* (2011) Phagostimulatory effect of uptake of PLGA microspheres loaded with rifampicin on alveolar macrophages. *Colloids Surf., B: Biointerfaces*, **87**, 293–298.
[17] Kalluru, R., Fenaroli, F., Westmoreland, D. et al. (2013) Poly(lactide-co-glycolide)-rifampicin nanoparticles efficiently clear Mycobacterium bovis BCG infection in macrophages and remain membrane-bound in phago-lysosomes. *J. Cell Sci.*, **126**, 3043–3054.
[18] Clemens, D.L., Lee, B.Y., Xue, M. *et al*. (2012) Targeted intracellular delivery of antituberculosis drugs to Mycobacterium tuberculosis-infected macrophages via functionalized mesoporous silica nanoparticles. *Antimicrob. Agents Chemother.*, **56**, 2535–2545.
[19] Pham, D.D., Fattal, E. and Tsapis, N. (2015) Pulmonary drug delivery systems for tuberculosis treatment. *Int. J. Pharm.*, **478**, 517–529.
[20] Tsapis, N., Bennett, D., Jackson, B. *et al.* (2002) Trojan particles: large porous carriers of nanoparticles for drug delivery. *Proc. Natl. Acad. Sci. USA*, **99**, 12001–12005.
[21] Sung, J.C., Padilla, D.J., Garcia-Contreras, L. *et al*. (2009) Formulation and pharmacokinetics of self-assembled rifampicin nanoparticle systems for pulmonary delivery. *Pharm. Res.*, **26**, 1847–1855.
[22] Balducci, A.G., Cagnani, S., Sonvico, F. et al. (2013) Pure Insulin Highly Respirable Powders for Inhalation. *Eur. J. Pharm. Sci.* doi:10.1016/j.ejps.2013.08.009.
[23] Hoppentocht, M., Akkerman, O.W., Hagedoorn, P. *et al*. (2015) The Cyclops for pulmonary delivery of aminoglycosides; a new member of the Twincer™ family. *Eur. J. Pharm. Biopharm.*, **90**, 8–15.
[24] Young, P.M., Crapper, J., Philips, G. *et al*. (2014) Overcoming dose limitations using the Orbital(®) multi-breath dry powder inhaler. *J. Aerosol Med. Pulm. Drug Deliv.*, **27**, 138–147.
[25] Newhouse, M.T., Hirst, P.H., Duddu, S.P. *et al*. (2003) Inhalation of a dry powder tobramycin PulmoSphere formulation in healthy volunteers. *Chest*, **124**, 360–366.
[26] Hoppentocht, M., Hagedoorn, P., Frijlink, H.W., and de Boer, A.H. (2014) Developments and strategies for inhaled antibiotic drugs in tuberculosis therapy: a critical evaluation. *Eur. J. Pharm. Biopharm.*, **86**, 23–30.

11

Inhaled Drug Combinations

Sanketkumar Pandya, Anuradha Gupta, Rajeev Ranjan, Madhur Sachan, Atul Kumar Agrawal and Amit Misra

CSIR Central Drug Research Institute, Lucknow, India

11.1 Introduction

As monotherapy is contra-indicated in the treatment of tuberculosis (TB), treatment regimens must include multiple drugs, hitting a variety of drug targets. Inhaled therapies proposed for TB include both single- and multiple-drug (fixed-dose combination; FDC) formulations. Formulations containing either first-line or second-line anti-TB agents are being studied by a number of research groups as either as stand-alone interventions or as adjunct therapies. Combinations of two or more first-line drugs; first-line drugs with experimental agents such as fluoroquinolones or rifamycins other than rifampicin; and with second-line drugs have been proposed. Drugs that inhibit resistance mechanisms of the pathogen, such as drug efflux pumps, are also proposed as candidates for inclusion in combination therapy of multiple-drug-resistant (MDR) TB. Finally, combining host-directed therapies with bactericidal agents has been proposed as an emerging strategy of a multi-pronged approach to shorten the duration of treatment of TB. Inhaled drug formulations are designed with one of two primary objectives: (*a*) total replacement of a conventional method of administration to achieve comparable blood levels, or (*b*) targeting lung macrophages and parenchyma without regard to blood levels. Inhaled particles also serendipitously serve a third objective: 'classical' or M1 activation of 'alternatively' activated or M2 cells harbouring the bacteria. While FDC inhalations offer the advantages of convenience to the patient, there are challenges in pulmonary delivery of doses to attain bioequivalence in

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

terms of blood levels of two or more drugs. Micro-doses of macrophage-targeted particles containing combinations are less challenging and appear to offer additional opportunities for novel combinations, including host-directed therapies.

11.2 Standard Combinations in Oral and Parenteral Regimens

11.2.1 Combinations for the Directly Observed Treatment Short-Course (DOTS) Regimen

The World Health Organization's current treatment guidelines [1] recommend a two-month intensive phase of treatment of new patients of both pulmonary and extra-pulmonary TB with a combination of isoniazid (H), rifampicin (rifampin, R), pyrazinamide (Z) and ethambutol (E). This is followed by four months of treatment with a combination of H and R. The six-month regimen is referred to as 2HRZE/4HR. Streptomycin (S), which must be administered parenterally, is recommended in place of E, especially in cases of tuberculous meningitis. The guidelines also recommend that the old regimen of 2HRZE/6HE should be discontinued in view of the higher relapse rates associated with its use.

The primary rationale for combination drug therapy of infectious diseases lies in the advantages inherent in attacking the pathogen at multiple targets. Thus, the components of the HRZE regimen target cell wall mycolic acid synthesis, DNA-dependent RNA polymerase, ribosomal protein S1 (RpsA) and arabinogalactan synthesis, respectively. This multiplicity of targets may be expected to result in synergy, but a surprisingly small number of studies have documented fractional inhibitory concentration indices (FICI) of these agents against *Mycobacterium tuberculosis* (Mtb). Perhaps equally surprisingly, synergy is not commonly observed in three-dimensional chequerboard designs. For instance, H when combined with R showed a FICI of 1.01 against a drug-sensitive, wild-type clinical isolate and 0.94 against the laboratory strain H37Rv. In combination with E, these values were 1.1 and 1.08, respectively [2]. In another study, there was no significant difference between FICI against drug-susceptible and MDR strains [3]. Recalling that synergy may be inferred from FICI values < 0.75, it may be concluded that anti-TB activities of these agents are additive and not synergistic. However, the ethylenediamine-based candidate drug SQ109 is strongly synergistic with R, even though its 'parent' drug E is not [4].

The doses of the five so-called first-line drugs are recommended for patients of body weights 30–39 kg, 40–54 kg, 55–70 kg and >70 kg. Thus, oral drug intake by a 60-kg patient during 2HRZE is about 3.6 grams a day. To reduce 'pill burden' imposed on the patient by the high dose of anti-TB drugs, and also to facilitate 'direct observation' of drug intake, FDCs have been recommended by the WHO and adopted by several national programmes. Clinically, there is no difference in the outcomes of treatment with FDC or with individual tablets [1], but a pharmaceutical curiosity has been reported in the literature since 2005. In a detailed study of drug–drug interactions among the first-line anti-TB drugs, Singh a nd co-workers observed that mixtures containing H and/or E showed up to 95% depletion of H and R under storage conditions of 40 °C/75% relative humidity within 90 days [5]. H is an acid hydrazide, expected to interact with any or most of the carbonyl groups present in R and other rifamycins by the well known hydrazone reaction [6], as depicted in Figure 11.1. Adducts of H and R were patented as anti-TB compounds in the 1970s [7], [8]. We [9] and

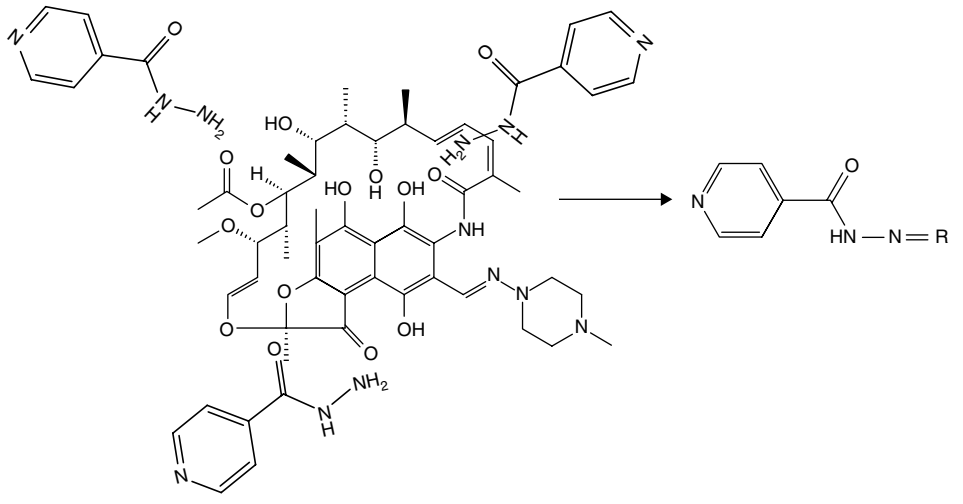


Figure 11.1 *H (isoniazid) can potentially interact with carbonyl groups present on R to yield a hydrazone.*

others [10–12] have reported a drug–drug incompatibility between H and R under acidic conditions similar to those found in the stomach, and formulation approaches to limiting this interaction [13].

Bioequivalence studies conducted using FDC and individual drugs have demonstrated equivalence in terms of area under the plasma concentration versus time curve (AUC) and maximum drug concentration in plasma (C_{max}) in respect of some formulations and settings [14, 15], but not in others [16, 17]. As recommended recently by Alsultan and Peloquin, therapeutic drug monitoring (TDM) is likely the best way to understand drug–drug incompatibility in real-life treatment of TB [18]. However, TDM for DOTS patients is not likely to be implemented in the field any time soon.

11.2.1.1 Combinations for Alternative Regimens Treating Drug-Sensitive and Drug-Resistant TB

Drug combinations addressing ease of use, amelioration of toxicity and shortening of duration are suggested by recent clinical results on experimental regimens. Thus, it might be prudent to design combinations that can dovetail with the objectives of interest of clinical investigations that are in progress, especially if the dosing patterns, pharmacokinetics and biodistribution can be linked to efficacy.

Several alternative regimens are under preclinical and clinical investigation. The most difficult, but also the most attractive, objective of research on alternative regimens is to come up with a multi-drug therapy that does not use either H or R. If this is achieved, it would obviate the need to undertake drug-sensitivity testing of bacteria recovered from patients to check for resistance to H and/or R. Other studies aim to replace H, R, Z or E in the intensive phase of treating TB. Zumla *et al.* summarized combination regimens under advanced preclinical and clinical evaluation in 2013. Ten classes of regimens were

identified; namely, those containing: high-dose rifamycins (rifapentine, R) or H; fluoroquinolones (gatifloxacin, moxifloxacin); bedaquiline; delamanid, oxazolidinones (linezolid, sutezolid), rhimnopterazines (clofazimine) and AZD8547 [19, 20]. Dose-finding studies with rifamycins and H are welcome, since it is now acknowledged that the doses used in DOTS are not derived from any clinical evidence.

The fluoroquinolones gatifloxacin and moxifloxacin have been studied clinically. A Cochrane systematic review in June 2013 concluded that there was insufficient evidence to conclude whether or not these fluoroquinolones were non-inferior to the HRZE regime [21]. Jawahar *et al*. reported soon after that intermittent (three times a week) administration of moxifloxacin or gatifloxacin in the intensive phase had to be discontinued if Z and/or E were withdrawn, since relapse rates rose [22]. Their study has been critiqued in PubMed Commons and by Zumla *et al.* for not running the different treatment arms concurrently, and for not commenting on an apparent 10% increase in sputum-converted patients at the end of the first two months, but it is noteworthy that the results do not contradict OFLOTUB, REMOX and RAQUIN trials as summarized by Zumla *et al.* [19]. In a more recent report on OFLOTUB, Merle *et al.* were not able to demonstrate non-inferiority of the gatifloxacin-containing regimen, despite better patient compliance compared with the 2HRZE/4HR regimen [23]. The OFLOTUB team and others [24] have drawn attention to the fact that orally administered fluoroquinolones do not attain high drug levels in caseous lesions [25, 26].

Combining moxifloxacin with intermittent high-dose (900 mg) rifapentine over six, but not four, months was equivalent to 2HRZE/4HR [27]. Four-month regimens in which moxifloxacin replaced ethambutol were also inferior to the standard regimen in a different trial [28]. This result does not detract from the observation that the early bactericidal activities (EBAs) of combinations containing fluoroquinolones are higher than that of HRZE. Five combinations of TMC207 (bedaquiline), PA-824, moxifloxacin and pyrazinamide were compared with that of an FDC containing H (75 mg), R (150 mg), Z (400 mg) and E (275 mg) in patients of drug-sensitive TB [29]. Encouragingly, one of the four-drug regimens lacking H and R was clearly non-inferior to the HRZE regimen.

Lienhardt *et al*. have suggested a 'new model' for clinical development of combination therapy [30]. Expanding this model to include additional details, it is possible to arrive at a pharmaceutical product development road map to registration, as shown in Figure 11.2. There is urgent need for such product development research to address drug combinations, especially since the emerging consensus clearly acknowledges that even new drug discovery will have to dovetail with a multi-drug, prolonged-use regimen.

While the majority of development efforts addressing drug combinations are likely to focus on orally administered formulations, we submit that pulmonary TB is best treated using pulmonary drug delivery [31, 32].

11.3 The Rationale for Inhaled Therapies of TB

Four of the five principal classes of drugs used for pharmacotherapy of asthma till the turn of the 20th century had a 5000-year history of empirical medicine, and were principally administered orally [33]. Asthma is a disease of the airways, but it took a lot of time for best practices to adapt to inhalation therapy of asthma. At this time, inhalations are routinely

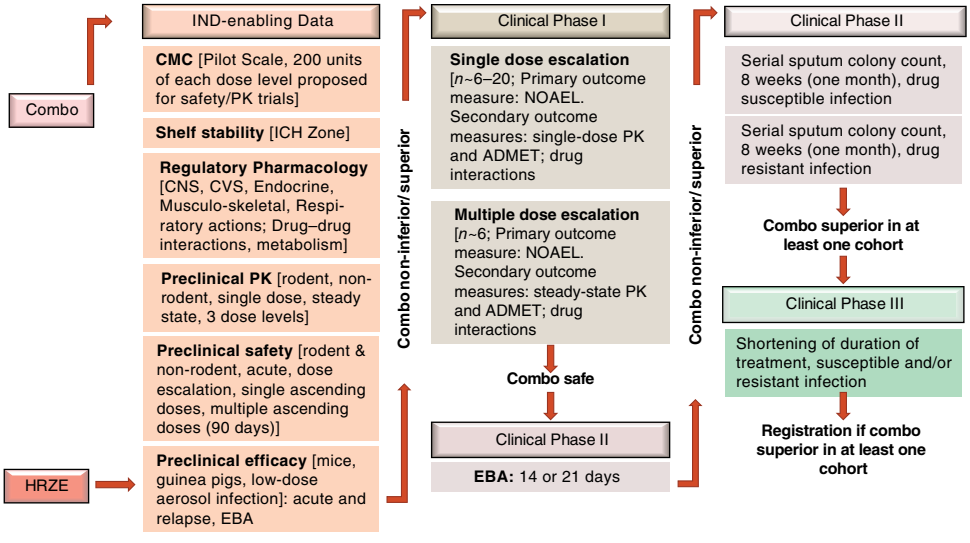


Figure 11.2 *Generic product road map for developing drug combinations for treatment of drug-sensitive or drug-resistant TB*

prescribed for asthma. Standard medication consists of an inhaled corticosteroid and a long-acting beta-2 receptor antagonist, both at microgram dose levels. Physicians and patients are by now comfortable with the idea of inhaling a few milligrams of material containing a few hundred micrograms of medication. This apparent comfort zone appears to be working both positively and negatively towards building opinion on inhaled therapies for TB.

TB is a lung disease, and the principal target of pharmacotherapy is the pathogen. Several hundred milligrams of anti-TB drugs are administered orally and repeatedly as described above. The 'deeper' location of the drug target and the order-of-magnitude higher drug dose does much to inspire doubt as to whether inhaled therapies for TB can 'work' [34].

The case for high-dose, deep-lung delivery of anti-TB agents has been made out elsewhere in this volume. Here, it is important to appreciate the importance of inhaled drug combinations, particularly in macrophage-targeting, particulate form.

Several researchers have suggested that the drug–drug incompatibility between R and H may be easily overcome if the two drugs are incorporated individually in two different particles, and the resultant powders mixed in appropriate proportion. To one way of thinking, this approach seems statistically feasible yet stochastically flawed. Considering Figure 11.3, it may be argued that although the statistical probability of each infected macrophage picking up an equal number of 'red' particles and 'green' particles is the same, the stochastic process of phagocytosis may actually result in different macrophages taking up different numbers of the two particles. This might result in a finite number of infected macrophages being subjected to monotherapy. Compounding this scenario, depleting concentrations of the drug incorporated individually in one kind of particle may, over time, select for drug-resistant mutants. Both these adverse outcomes are avoided if the two drugs are incorporated in the same particle. Regardless of the number of particles that a macrophage picks up, both drugs would be present in the cytosol; ideally at the ratio of their

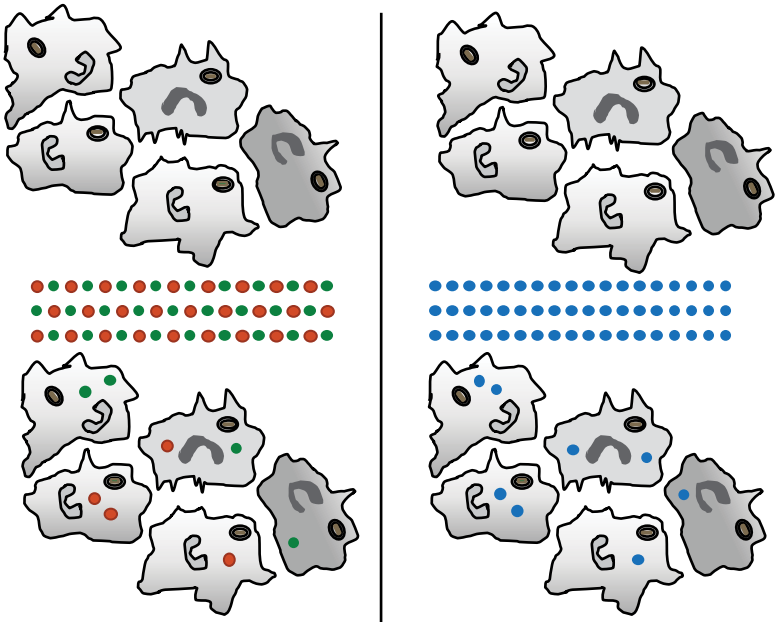


Figure 11.3 *Statistical versus stochastic uptake of particles by infected macrophages. (See insert for color representation of the figure.)*

optimal bactericidal concentrations if drug-release rates are controlled through particle formulation approaches.

There are several reports on pulmonary delivery of anti-TB drugs, either supplementing the standard dosing regimens or replacing them as a whole. Some of the objectives of administering inhalations include targeting macrophages harbouring bacteria, aiming for increased concentration in and around granulomas so that drugs may diffuse through the physical barrier surrounding it, higher bioavailability, and reduced systemic toxicity. Migration of macrophages to the inaccessible regions in the lungs is suggested by studies in the zebrafish embryo, wherein fluorescence-labelled particles taken up by macrophages trafficked to the site of infection [35]. These individual objectives require a closer look.

11.3.1 Single Drug, Supplementing Other Orally Administered Drugs

Supplemental or adjunct inhaled formulations to be administered along with the standard oral regimen have been proposed by a number of research groups since O'Hara and Hickey [36]. Formulations containing R alone are the most numerous in the literature [37–56]. R is a drug of choice for formulation scientists for several reasons; but primarily because it is readily soluble in processing solvents, has a minimum inhibitory concentration (MIC) of 0.1–0.2 μg/ml against intacellular Mtb H37Rv [57], and displays exposure-dependent (concentration × time) kill kinetics against Mtb in macrophages [58]. R has demonstrated a sterilizing effect in animal models, an important attribute to prevent transmission of the disease from patients to other individuals in the population [59]. R has a half-life of 2.5 hours and it is 85% protein bound. Inhalable polymeric particles releasing R in a

controlled manner would be able to maintain high drug concentrations in the macrophage cytosol for a long duration, increasing the efficacy.

Inhalable formulations of H alone are rarer [60–65]. More work in this area is required, since H has the highest EBA among all drugs in the standard regimen, and pulmonary delivery of controlled-release H to infected macrophages can potentially target the pathogen in the environment that the bacteria find most conducive for replication. Inhalable formulations of Z alone have only begun to be reported recently [66, 67], and formulations containing its active metabolite, pyrazinoic acid, have been proposed [68]. Inhalation formulations containing E alone have not been reported in the public domain to our knowledge. The high MIC value and the lack of an ultraviolet chromophore in E for easy quantitative analysis are apparent hindrances in research efforts aimed at developing inhalations to replace oral E in the standard regimen.

The anti-TB agent *p*-aminosalicylate has been formulated for pulmonary delivery to achieve targeted drug delivery to the lung and reduce the total quantum of dose [69, 70].

11.3.2 Single Drug Replacing Injectable First- or Second-Line Agents

Injections require trained personnel for administration, are distressful for the patient, and are associated with additional complications in resource-poor settings. Streptomycin, kanamycin, amikacin, and capreomycin constitute the group of injectable drugs used for the treatment of TB. S is a first-line drug, either prescribed to treat patients that have been treated for TB in the past to reduce the chances of drug resistance, or sometimes substituted for E. The remaining drugs are second-line agents, used in treatment regimens of MDR-TB involving 5 drugs. Aerosol inhalation of streptomycin was first reported as early as 1959 [71]. However, this and later reports from Russia remain inaccessible, and concerns have been raised about the quality of data reported by them. Early reports from Japan on clinical use of inhaled streptomycin are also equivocal. For instance, Rikimaru *et al.* reported clear benefits of nebulized streptomycin administered daily over 28 days in tracheobronchial TB, but sample-size limitations did not permit a clear demonstration of benefit in patients with pulmonary TB [72].

Rapid sputum conversion within 23 days was reported in patients with refractory TB, whether or not the infection was drug resistant, upon add-on therapy with nebulized aminoglycosides kanamycin or gentamycin [73]. A liposomal formulation of kanamycin has also been reported [74]. A retrospective study on refractory non-tuberculous mycobacterial (NTM) infections in patients receiving amikacin by pulmonary administration in addition to other regimens found improvement in symptoms and bacterial burden [75]. A liposomal formulation of amikacin is reported to show about 25% higher efficacy in a mouse model of TB [76]. Moxifloxacin and ofloxacin inhalable powders prepared by spray drying along with dipalmitoylphosphatidylcholine (DPPC) have been reported [77]. Moxifloxacin has also been reported as a chitosan-based inhalation formulation for controlled drug release and enhanced interaction of the delivery system with lipid bilayers [78]. Chitosan [79] and hyaluronan [80] particles containing ofloxacin for pulmonary delivery have been reported as well. Levofloxacin proliposomes are also under investigation [81].

The experimental drug PA-824 was formulated in lipid-based porous particles for systemic delivery through the pulmonary route [82]. However, efficacy of inhaled PA-824 was tested at fairly low inhalation doses and was lower than that of an orally administered suspension [83].

Capreomycin is the most advanced inhaled therapy under investigation as part of an anti-MDR regimen, having successfully completed Phase I human trials [84]. Details are discussed elsewhere in this volume. Other pulmonary delivery formulations of capreomycin have been reported [85, 86]. The water-insoluble second-line anti-TB drug isoxyl has unfortunately been clinically tested after oral delivery, but may instead be formulated as a dry-powder inhalation [87].

11.3.3 Multiple Inhaled Drugs, Adjunct or Stand-alone Therapy

Table 11.1 lists some inhalable formulations containing drug combinations. It is apparent that formulation researchers perceive the potential of pulmonary drug delivery not only as an option for administering low doses for adjunct therapy, but also as a stand-alone platform. Thus, seven of 11 combination formulations reported at the time of writing can be discerned as intended for inhaled therapy without additional oral dosing. Further translation research on these combinations is not reported in the public domain.

The debate on adjunct versus stand-alone inhaled therapies receives inputs from different areas of expertise. There is limited experience in clinical use of high-dose inhalations, and, till recently, there was scepticism as to whether several hundred milligrams could be delivered by inhalation. The capreomycin trial has clearly demonstrated that 300 mg of capreomycin, at least, can be safely inhaled [84]. The utility or even benefit of sustained drug release in the lung may sometimes have been over-emphasized in the early literature, and the perils associated with neglecting plasma concentrations in view of extra-pulmonary TB might have been correspondingly downplayed. Finally, it has been pointed out that vigorous pursuit of inhaled therapies as 'mere' adjuncts to standard oral dosing could constrain clinical and policy opinion to regard these as inadequate for stand-alone application. For the formulation-development community, it might be wise to pursue both the adjunct and the stand-alone streams equally diligently. While it certainly seems possible that adjunct therapies involving low drug doses have a better chance of finding regulatory and ethical approval for clinical testing, the full potential of pulmonary drug delivery will be realised when four- or five-drug regimens can be delivered by inhalation.

11.3.4 "Stimulate the Phagocyte"

The phrase above is attributed to Eli Metchnikoff, but has been used to describe a therapeutic approach that looks upon macrophages through the eyes of the Fabian/Marxist thinker George Bernard Shaw [98]. This literary allusion contrasts disruptive climaxes often seen in Shaw's plays with a more ambitious objective – decreasing inflammatory macrophage responses that can give rise to immunopathology, even as bactericidal responses are retained. Such subtle, patient-specific immunomodulation is not easily achievable at this time, but there is certainly a case for closer evaluation of drug combinations that rescue Mtb-infected macrophages and dendritic cells from alternative [99], M2 [100] or mixed M1/M2 [101] activation brought about by the pathogen. Pharmacological agents that can do so are considered in greater detail in the section on host-directed therapies below, but given that anti-TB agents themselves have immunomodulatory potential [98], there is legitimate scope for inhaled therapies that target particles or vesicles to Mtb-infected lung macrophages to base their rationale upon stimulation of host macrophage responses [102, 103].

Table 11.1 *Inhaled combinations reported up to November 2014*

Drugs	Excipient	Administration	Objectives	Remarks[a]	Ref.
HR	Gelatin	Neb[b]	Formulation	—	[63]
HR	*poly*(L-lactic acid)	DPI[c]	Targeting lung macrophages	A	[88]
H + rifabutin	*poly*(L-lactic acid)	DPI	Targeting, avoiding drug incompatibility, reducing inhaled dose	A	[89]
HRZ	*poly*(DL-lactide-co-glycolide)	Neb	Improving systemic bioavailability, reducing dose	A	[90]
HRZ	Stearic acid	Neb	Improving bioavailability and reducing dosing frequency	A	[91]
HRZ	Alginate	Neb	Controlled release of drugs	A	[92]
HRZ	Excipient free	DPI	Rapid and efficient resolution of local and systemic infection	S	[93]
HRZ	Lactose	Neb	Targeting lung tissue, dose reduction	S	[94]
HRE	DPPC	Neb	Targeting lung macrophages	A	[95]
HRZ + ethionamide + streptomycin	DSPC[d]/cholesterol	—	Increasing therapeutic index	S (Poor incorporation of R, streptomycin, ethionamide	[96]
Z + rifapentine + moxifloxacin	Leucine	—	Target lungs, avoid plasma protein binding	A	[97]

[a] A = Adjunct therapy; S = Stand-alone therapy.
[b] Nebulizer.
[c] Dry-powder inhaler.
[d] Distearoylphosphatidylcholine.

11.4 Combinations of Anti-TB Drugs with Other Agents

A variety of drugs that are not themselves active in killing Mtb can be fruitfully combined with anti-tuberculosis therapies. These drugs may be used to interfere with the physiology of either the pathogen or the host. The therapeutic rationale of employing such drugs resides in acknowledging that infectious disease cannot be viewed merely as an outcome of the presence of the pathogen – the host is an equally important component of pathogenesis. Thus the host–pathogen dialectic is itself a 'drug target' in a wider sense of the term. However, in the interests of developing a rigorous understanding, it may be helpful to temporarily separate pathogen-directed strategies from those that are host-directed. Such an approach can enable a better appreciation of how drugs can be 'repurposed' or 'repositioned' for use as adjunct therapy of TB.

11.4.1 Drugs that Primarily Affect the Pathogen

Realizing that drug efflux pumps represent a primary mechanism of drug resistance exhibited by Mtb strains, efflux-pump inhibitors have been emphatically proposed as components of treatment regimens in MDR/XDR (extensively drug-resistant) TB. For instance, verapamil has been proposed as an adjunct to standard regimens to combat drug-resistant TB [104]. Verapamil interferes with mycobacterial efflux pumps, but also results in decreased permeability of calcium channels, reduced flux of calcium from the macrophage cytosol, generalized vasodilation and low-level anti-inflammatory effects [105–107]. In the so-called Kramnik mouse model infected with drug-susceptible Mtb H37Rv, addition of 9.4 mg/kg of verapamil to a regimen comprising 2HRZ/4HR resulted in statistically significant differences in lung bacterial burden from the first week itself. Verapamil speeded up the time to culture conversion by about a whole month and no relapse was observed [105]. More recently, it was demonstrated that drug-resistant strains were rendered susceptible to the cognate drug in a cell culture model of infection. Even more encouragingly, the *R*-isoform of verapamil and verapamil's primary metabolite norverapamil, which have significantly lower Ca^{2+} channel-blocking activity and therefore lower potential cardiotoxicity, are equally efficient at reversing drug resistance [108]. Research is also underway to synthesize and test analogues of verapamil for use in TB [109].

To keep the host–pathogen dialectic in perspective, it is instructive to recall that the efflux pumps that mediate drug resistance are induced in Mtb during the process of the bacteria adapting to the intracellular environment of the macrophage [108, 110]. From the perspective of lung macrophage-targeted inhaled therapies, it is interesting to note that a large number of potential toxicity issues and considerations relating to off-target drug actions of Ca^{2+} channel blockers can be addressed by an inhaled formulation containing a combination of at least two potent anti-TB drugs with verapamil. Such a formulation would also be able to avoid the necessary escalation of verapamil dose when co-administered orally with R on account of their drug–drug interaction [105, 111]. Thus, it is submitted that macrophage-targeted drug delivery by inhalation may be the safest and most effective way to deploy the therapeutic strategy of inhibiting macrophage-induced efflux pumps in Mtb infection. Work on developing such a formulation is in progress in the authors' lab.

The neuroleptic agent thioridazine is another efflux-pump inhibitor/ion-channel blocker that was reported to overcome Mtb drug resistance several years ago [112]. Other

phenothiazines were tested against clinical MDR isolates, and chlorpromazine was identified as being equipotent to thioridazine. However, MIC_{50} values were considered too high to be able to be achieved clinically, and the authors commented on macrophage accumulation of these agents while recommending them as adjunct therapy [113]. Mouse studies at two independent locations, however, have demonstrated clear advantages of including thioridazine in regimens for both drug-sensitive and -resistant MDR [114, 115]. Yet again, incorporation of thioridazine in a lung macrophage-targeted inhaled formulation can offer promise of dealing with off-target effects and the dose requirements of phenothiazines.

NADH:menaquinone oxidoreductase activity and host-cell calcium and potassium channels can also be used as salvage therapy in MDR-TB [116, 117]. Moreover, reserpine, an ATP-dependent efflux-pump inhibitor, has been shown to enhance the efficacy of ciprofloxacin and linezolid against Mtb strains [118, 119]. Piperine, an efflux-pump inhibitor of Rv1258c, increased the efficacy of R by 4- to 8-fold both in sensitive and R-resistant Mtb [120].

11.4.2 Drugs that Affect Host Responses

While possessing no intrinsic activity against Mtb itself [121], 1,25-dihydroxyvitamin D3 is a host metabolite that has often been proposed as an agent that contributes to chemotherapy of TB. It is thought to contribute towards induction of bactericidal macrophage responses, and has recently been demonstrated to participate in regulation of lipid metabolism in the infected macrophage [122]. A meta-analysis of clinical studies on vitamin D in TB revealed that in studies that report sputum or smear culture results, there was an aggregate reduction of 39% in the risk of remaining culture positive after six weeks of receiving vitamin D supplementation along with the standard regimen. This risk reduction, however, was not statistically significant [123]. Another meta-analysis, this time with studies involving vitamin D supplementation excluded from the analysis, suggested that although strong evidence for association between the vitamin D status of the patient and disease status is still lacking, this may be due to study designs that do not measure the status of infection as a primary outcome measure [124].

Other drugs that affect lipid metabolism in the infected macrophage include statins such as atorvastatin [125] and the drug candidate SPR-113 [126].

Autophagy [127] and/or apoptosis [128] of the Mtb-infected macrophage have been clearly implicated as host macrophage responses that can work against Mtb infection [129]. Anti-TB activity of inhalable particles containing rapamycin, the normative inducer of autophagy, has been demonstrated *in vitro* [130] and mouse experiments are underway to evaluate whether inhalations of particles containing rapamycin along with H and rifabutin are non-inferior to inhalations of the anti-TB agents alone. Some reports of inhaled therapies that induce apoptosis in the infected macrophage are available [131–133], and there are indications that such induction is caspase-independent [131, 134].

There are other experimental agents that have the potential to combine with inhaled therapy of TB via actions restricted primarily to the host cell. These include muramyl dipeptide for generalized immunostimulation of alveolar macrophages [135, 136], small interfering RNA (siRNA) targeting the human chemokine XCL-1 [137], the pathogen-favouring cytokine TGF-β [138], TNFα [139] and suppressors of cytokine signalling (Mohan *et al.*, unpublished).

11.4.3 Drugs that Affect both Host and Pathogen

Nitric oxide (NO) is an effector molecule capable of killing Mtb through generation of reactive nitrogen intermediates that nitrosylate bacterial proteins, including respiratory enzymes [140]. NO is also a signalling molecule with diverse functions relevant for the activation status of the infected macrophage. However, human macrophages do not produce bactericidal concentrations of NO and the amounts that they do produce may even benefit the survival of intracellular Mtb [141]. Experiments on inhalable microparticles containing diethylenetriamine–nitric oxide adduct (DETA/NO) or sodium nitroprusside (SNP), either alone or in combination with H and rifabutin, have demonstrated that exogenously supplied NO-donor is bactericidal and additive in anti-TB effects, re-establishing phagosome–lysosome fusion and acidification [142, 143].

Nitazoxanide and its metabolite tizoxanide are antiprotozoal agents, approved for use against amoebiasis and giardiasis. Nitazoxanide possesses appreciable activity against clinical MDR strains of TB [144]. It also induces autophagy in Mtb-infected macrophages and inhibits proliferation [145]. Investigations are currently underway to compare inhaled particles containing rapamycin with those containing nitazoxanide in a mouse model of TB.

Reasoning along lines illustrated above, it is possible to identify several candidate drugs for repurposing as adjunct therapies to TB – however, the off-target effects of these drugs may best be avoided and their doses optimally reduced if they are formulated as inhaled therapies targeting lung macrophages.

11.5 Formulation of Inhaled Drug Combinations

Although it has been reiterated *ad nauseam* that inhaled drug products are multi-component entities, it is worthwhile recalling once again that drug combinations add yet another variable to pulmonary drug delivery systems. Thus, the powder, suspension or solution containing the active pharmaceutical ingredients (APIs), the unit-dose container (capsule, blister), and the inhalation device (DPI, pressurized metered dose inhalers, nebulizer or other) have to be carefully and painstakingly matched to each other for optimal product performance. General principles that are universally applicable are not touched upon here, but some special cases relevant to anti-TB drugs are worthy of closer attention.

11.5.1 Excipient-free Formulations

The obvious and compelling advantage of excipient-free formulations is that the delivery of biologically inactive ingredients is curtailed. Son and McConville reported spray drying of R to make a flaky powder with good aerosol properties [49] and multiple-nozzle spray drying of an aqueous suspension of the particles together with an organic solution of *poly*(lactide-co-glycolide) polymer to obtain sustained-release particles [50]. While the first report describes an excipient-free formulation, the need to employ a release-retarding excipient was clearly articulated in the later report. Similarly, researchers based in several locations in Sydney report an inhalable form of rifapentine, in combination with moxifloxacin and pyrazinamide [146] or as amorphous or crystalline forms of rifapentine alone, intended as combination or adjunct inhaled therapy [147]. The three-drug combination was largely amorphous, but in the absence of leucine as a crystallization-inhibiting excipient,

showed a tendency towards crystallization of the pyrazinamide component. A human study on a dry powder containing H, R and Z prepared by trituration followed by mixing with 20 parts w/w of Inhalac 230 reported orders of magnitude higher concentrations of these drugs in the lung epithelial fluid lining and lung macrophages when compared with oral administration [94]. In the case of this powder, too, the use of excipient could not be eliminated, since the flow properties and aerosolization of the drug triturate were sub-optimal.

There is another factor that assumes importance in the design of excipient-free formulations of inhaled anti-TB drugs. Drug powders depositing in the alveoli will encounter a layer of lung surfactant, which is likely to be sufficient to rapidly dissolve known drugs. As a result of rapid dissolution, macrophage uptake of the deposited drug is likely to be compromised, since Fickian diffusion rather than phagocytosis would be the principal mechanism of uptake of drugs that enter solution. The formulation scientist therefore has to decide whether or not to opt for an excipient-free formulation in context of the divergent objectives of macrophage targeting and systemic delivery. In the absence of inactives that control dissolution behaviour and drug release, drug particles depositing on the lung surface will tend to distribute rapidly to the blood circulation, in proportion to their rates of permeation across the alveolar surface.

The foregoing considerations are illustrated well in the report of de Jesús Valle *et al*., wherein 400 µg of linezolid was administered in 5 ml of water to isolated rat lungs, either by perfusion or by nebulization [148]. Even though linezolid is only slightly soluble in water, the volume employed here was sufficient to achieve complete dissolution of the drug prior to nebulization. In contrast to the results of Katiyar *et al.* [94], the differences between intracellular and epithelial fluid concentrations achieved by inhalation were not different by orders of magnitude when a soluble drug was administered by the two routes.

It would appear, therefore, that excipient-free inhaled formulations are better suited to systemic delivery rather than targeting lung tissue, or more specifically, lung macrophages.

11.5.2 Applications of Excipients

Excipients are used for various applications: to improve powder flow, optimize aerosol generation and deposition, achieve macrophage targeting and/or sustained drug release in the lungs, and inhibit amorphous-to-crystalline transitions and, in the context of FDCs, to overcome drug incompatibilities.

The first two applications listed above are informed by a large body of work, and are not discussed here since there is no identifiable gap in the knowledge that is applicable to drug combinations. Targeting the lung macrophage is also a familiar objective, but it is instructive to note that many of the groups that aim to target these cells are actually taking advantage of a natural phenomenon. Lung macrophages have evolved to avidly take up particles depositing on the alveolar surface, so it is no surprise that particles that retain their particulate nature for sufficiently long periods after alveolar deposition are picked up by macrophages. From another perspective, with reference to the objective of using the lungs as a portal for systemic drug delivery, lung macrophages are something of a nuisance if the medicament is formulated as a powder that retains integrity for some time after lung deposition. In much of the literature, macrophage uptake of inhaled particles is referred to as macrophage clearance. In the non-tubercular lung, macrophages tend to take up particles and migrate towards ciliated airways, so that these foreign bodies may be

pushed upwards into the larynx. In a lung with a granuloma, however, some macrophages that take up particles may be chemo-attracted to the lesion. As reported by Fenaroli *et al.* [35], it is likely that a granuloma in the human lung could be surrounded by macrophages that have taken up drug-containing inhaled particles. Excipients that can promote such an outcome would be truly valuable, because the formation of a drug-laden cordon around the granuloma will not only promote reduction of the path length of diffusion that a drug molecule has to undertake in order to reach the lesion, it will also ensure that bacteria in the process of egress from the granuloma will encounter a shell of high drug concentrations that they might find difficult to emerge alive from.

Excipients that make use of ligands that engage host signalling receptors have been used in some formulations. Heuking *et al.* used chitosan with the awareness that it is a ligand for the human Toll-like receptor (TLR)-1 and TLR-2 [149]. To further enhance engagement with these 'danger' receptors, they functionalized chitosan particles containing an anti-TB DNA vaccine candidate with an agent that activates virtually all TLRs. They observed that engagement of TLRs by inhaled particles promoted an appropriate and robust immune response. Pulmonary or conventional vaccination requires very few doses as compared with the chronic regimens that chemotherapy requires. Concerns regarding long-term use of TLR ligands in drug inhalations are likely to arise, and would need to be empirically addressed. However, Ahmad *et al*. have used a similar polysaccharide, alginate, to prepare a pulmonary delivery formulation of H, R and Z and demonstrated its high efficacy and safety in the Guinea pig model [150].

Lactide-glycolide polymers have been used most commonly to make particles for macrophage targeting, since these are considered biocompatible and non-allergenic as well as biodegradable. However, we have observed that phagocytosis of particles composed of high-molecular-weight *poly*(L-lactide) with no drug content induces several markers of macrophage activation [102, 130, 151–153]. In contrast, several reports are available that drug-free or drug-loaded particles composed of *poly*(lactide-co-glycolide) of moderately high molecular weight are entirely benign. For instance, Hirota *et al.* found no elicitation of pro-inflammatory or immunosuppressive cytokines or even nitric oxide from macrophages when particles containing R in a *poly*(lactic-co-glycolic acid) (PLGA) matrix were added to cultured rat alveolar macrophages [47]. Thus, it falls to the formulation scientist to factor in the role of the excipient of choice in both targeting the macrophage and activating it upon phagocytosis of the particle.

The role of drug–excipient compatibility and the possibility of using excipients to control drug–drug incompatibility also require attention. The hydrazone reaction referred to above in the context of H and R [10] is likely to occur between H and the carbonyl groups present in keto- sugars like ribulose and fructose. The porphyrin nucleus of R is susceptible to chelating cations, and Fe^{+3} is reported to form a water-insoluble complex with R [154]. Fluoroquinolones are equally if not more suited for chelating divalent cations [155]. However, it is possible to use excipients to sequester incompatible drugs in different domains within the same particle. For instance, the anticancer agent 5-fluorouracil has chemotherapeutic synergy as well as drug incompatibility with folinic acid. Using β-cyclodextrin complexation to separate the two in the same formulation has provided the means to co-administer them in the same formulation [156]. Among the first-line anti-TB agents the following are well known to undergo complexation with cyclodextrins or similar host

molecules: H [157], R [158], and E [159]. Formulations containing R for nebulization [41] and as a spray-dried DPI [160] have been proposed. It would be interesting to investigate whether cyclodextrin complexation could inhibit incompatibility between H and R.

11.5.3 Preparing Multi-Component Particles and Vesicles

Spray drying is the most common technique reported for preparing inhalable formulations for TB therapy. There are several reasons why the method is increasingly finding use for preparing these formulations. The most attractive, in our view, is the industrial scalability of the process and the wide availability of necessary equipment. However, since the technique lends itself to using two-phase systems such as suspensions and emulsions as feedstock, there is a tendency among formulation scientists to pay less attention to the spray drying of true or molecular solutions. With respect to spray drying of feedstock that contains more than one drug, we believe that it is more desirable that all species should be present in their molecular state in order to ensure homogeneity of the particles obtained in the product. Solvent systems that solubilize drugs as well as excipients have been widely reported, and the effort required to arrive at an optimal solvent system is worthwhile. The option of mixing pre-formed solutions in suitable proportions is also viable. Sharma *et al.* [9] and Muttil *et al.* [89] reported spray drying a solution comprising H, R or rifabutin and *poly*(lactic acid) (PLA) that was formed by dissolving the rifamycin and polymer in dichloromethane, while H was dissolved in methanol. Addition 1 part of the methanol solution to 9 parts of the dichloromethane solution did not lead to precipitation.

Emulsion methods are a little more difficult to scale, and in the case of combinations wherein one component is freely water-soluble while others are not, significant loss of the water-soluble drug to the external aqueous phase is encountered. This loss may be inhibited by strategies such as the use of chilled glycerol as the external phase in which a drug–polymer solution is emulsified [36], but, in general, there is significant loss of anti-TB drugs when emulsion methods are used to prepare inhalable particles.

Supercritical-fluid methods, especially those employing supercritical CO_2 and co-solvents, are under active investigation as techniques to prepare inhalable particles for TB therapy. Polymeric particles incorporating R [40,161], ion-paired H [61] and *p*-aminosalicylic acid [162] have been reported, and techniques are reviewed elsewhere in this volume. With reference to drug combinations, however, not much is known about the use of appropriate co-solvents for drugs of differing solubility, although complex mixtures such as herbal extracts have been formulated as liposomes using supercritical fluids [163].

To our knowledge, there is no report of electrospray-mediated preparation of particles containing drugs for use in TB. However, the technique is simple, scalable and cost-effective, so investigations in the area would be welcome.

11.5.4 Shelf Stability

Drug product stability testing in respect of combinations is informed not only by the usual considerations of maintenance of identity, purity and performance criteria, but also by potential interactions that may occur between degradation products of one component and the remaining medicament. For instance, acid hydrolysis of H is likely to generate two chemically reactive entities as shown in Figure 11.4.

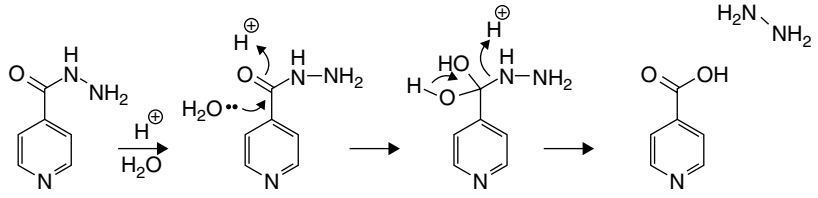


Figure 11.4 *Acid-catalysed hydrolysis of H to isonicotinic acid and hydrazine*

Hydrazine has been implicated in the decomposition of R in FDCs containing H and R [10] and might also affect other agents. Isonicotinic acid can also interact with a variety of anti-TB agents.

Even without degradation, Z and E are apparently capable of catalyzing the interaction between H and R [164]. Maintenance of storage stability during shelf life thus presents a variety of challenges. In the specific case of DPI, the greatest challenge to maintaining both chemical and performance stability lies in ensuring that moisture has negligible access to the powder. It may even be prudent to undertake manufacture and packaging in market-intended containers within dehumidified premises. The moisture that the product picks up on the shop floor is sometimes enough to compromise stability during storage.

11.5.5 Drug Release and Pharmacokinetics

Formulating sustained-release, pulmonary delivery particles containing drug combinations is challenging. The readily water-soluble H and poorly-soluble R incorporated in a polymer matrix are released at significantly different rates from PLA particles. Substitution of R by rifabutin leads to similar observations, including a time course of cytosolic concentrations in the mouse lung macrophage that is quite divergent for the first eight hours after inhalation, as shown in Figure 11.5 and reported earlier [165]. Data shown in Figure 11.5 re-invite questions about the advisability of incorporating two drugs in the same particle. In theory, a formulation scientist can design polymer matrices to incorporate the two drugs individually, and mix two or more powders in required proportions to arrive at the desired combination.

We have resisted the temptation to do this in the light of the scenario described in Figure 11.3 above, but that particular caveat is applicable only to formulations that seek to target the lung macrophage. Other kinds of formulations, designed for systemic drug delivery and able to evade macrophage uptake, may employ this strategy to advantage.

Studying drug release from powders containing more than one drug usually involves placing the powder in a dialysis bag and employing one of several pharmacopoeial and unofficial methods to sample the medium over desired lengths of time. A physical mixture of the components of the formulation, not subjected to the process of preparation of the particles, offers a useful control in these studies. Sometimes, one of the components of the formulation affects dialysis-membrane permeability, inducing artefacts such as unexpectedly long lag-time. In the absence of a control, it then becomes difficult to distinguish between formulations that genuinely show small levels of burst release, and others that do in fact show high burst release, but are masked by excipient effects on the semi-permeable membrane.

Similarly, prolonged drug release may be construed from the formulation itself in some cases, when the actual cause of retardation of drug release to extraordinary periods may lie

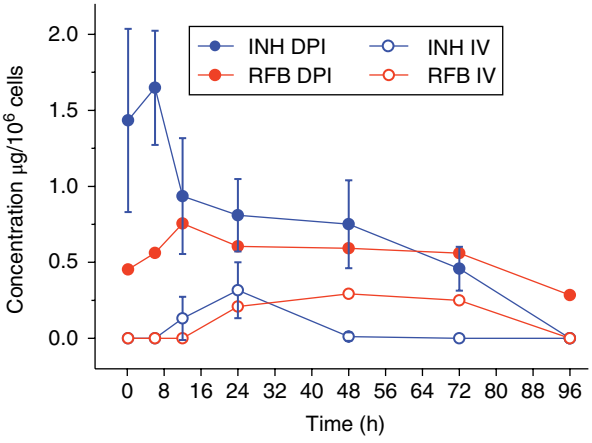


Figure 11.5 *Time course of isoniazid (INH) and rifabutin (RFB) in cells recovered by bronchoalveolar lavage from mice receiving about 100 µg of a dry-powder inhalation comprising 1:1:2 parts of isoniazid, rifabutin, and PLA or intravenous injections of the same amounts of drugs. (See insert for color representation of the figure.)*

in occlusion of the membrane by excipients. Donnan's membrane equilibrium and its influence on diffusion of solutes as well as water across semi-permeable membranes requires attention in designing these studies [166].

Pharmacokinetics and biodistribution of inhaled drugs are evaluated with regard to the objectives of pulmonary drug delivery as described above. If the intent is to retain the drugs in lung parenchyma or macrophages, tissue distribution and cytosolic concentrations as a function of time assume greater importance than do plasma concentrations. Plasma concentrations, however, need to be evaluated even if the objective is to deliver drugs for retention in the lungs. These data serve to validate the claim of lung targeting, especially if compared with doses delivered orally or parenterally. The use of a "çharcoal block" to inhibit gastrointestinal absorption of drugs deposited in the mouth is useful to distinguish the contribution of inhaled doses to blood levels [167]. With reference to combinations, evaluation of tissue and blood pharmacokinetics is sometimes complicated by significant differences in half-lives or mean residence times of multiple inhaled drugs. The combination of H with rifabutin was therefore evaluated by sparse sampling over 96 hours in order to allow the long-lived rifabutin to convincingly enter the elimination phase [165]. Finally, since TB requires chronic chemotherapy, steady-state pharmacokinetics and tissue distribution requires attention [168].

11.5.6 Inhalation Dosimetry

Estimation of doses inhaled by test animals and humans through the use of various formulations and devices requires painstaking effort. The most convincing evidence of such dose-determination studies is offered by 'material balance' studies, in which radiolabelled compounds are estimated in a large number of tissue compartments. The technique of pharmacokinetic 'bridging' can offer a shortcut to such dosimetry if the intravenous pharmacokinetics are well established. 'Bridging' may be accomplished in various ways.

Subcutaneous doses were 'bridged' with a standard intravenous dose based on readout of plasma concentrations at different times following test doses administered subcutaneously [169]. With respect to the inhaled combination of H and rifabutin targeting lung macrophages, 'bridging' was carried out in a slightly different manner. Using data available on disposition of intravenous radiolabelled rifabutin in a large number of tissues [170], we derived linear relationships between tissue concentrations at any time and the corresponding plasma level. We then administered doses to mice by intravenous, oral and pulmonary routes. Blood and tissue samples were then drawn, and the concentrations estimated in serum, liver, lungs and spleen were statistically compared with the values obtained by Battaglia et al. [170]. Confirming concordance between published and determined values in respect of these tissues, we then derived drug concentrations in other tissues by using the same linear relationship. By this exercise, we could establish tissue concentrations after intravenous and oral dosing with an accuracy of 96–119%. Assuming similar accuracy after pulmonary delivery of drug particles, we were able to establish the inhaled dose from a much smaller number of biological samples [171].

While such indirect approaches to inhalation dosimetry in preclinical studies may provide useful pointers, dose delivery is much more precise if pulmonary administration is undertaken using endotracheal intubation and positive-pressure insufflation. The Penn–Century devices available commercially for solutions and dry powders are extremely popular among researchers wishing to control the amount actually administered. However, there are several caveats to the use of this approach, especially with reference to dry powders [172], and appreciation of the fact that lung deposition under conditions of ambient-pressure inhalation is likely to differ significantly from forced deposition.

11.6 Conclusions

Multiple-drug therapy being a necessity in TB therapy, drug combinations could be a useful method to reduce pill burden. With special reference to inhaled therapies for TB, combinations of multiple drugs targeting the pathogen, as well as pharmacological and immunomodulatory agents that evoke beneficial host responses, are likely to be optimally delivered by macrophage-targeting inhalations. Appreciation of the behaviour of various drugs in proximity to each other is especially useful in formulating combinations that work as expected.

References

[1] WHO (2010) Treatment of tuberculosis: Guidelines. World Health Organization.
[2] Bhusal, Y., Shiohira, C.M. and Yamane, N. (2005) Determination of in vitro synergy when three antimicrobial agents are combined against Mycobacterium tuberculosis. *Int. J. Antimicrob. Agents*, **26**, 292–297.
[3] Rey-Jurado, E., Tudo, G., de la Bellacasa, J.P. *et al.* (2013) In vitro effect of three-drug combinations of antituberculous agents against multidrug-resistant Mycobacterium tuberculosis isolates. *Int. J. Antimicrob. Agents*, **41**, 278–280.
[4] Reddy, V.M., Einck, L., Andries, K. and Nacy, C.A. (2010) In Vitro Interactions between New Antitubercular Drug Candidates SQ109 and TMC207. *Antimicrob. Agents Chemother.*, **54**, 2840–2846.
[5] Bhutani, H., Singh, S. and Jindal, K.C. (2005) Drug-drug interaction studies on first-line antituberculosis drugs. *Pharm. Dev. Technol.*, **10**, 517–524.

[6] Dirksen, A., Dirksen, S., Hackeng, T.M. and Dawson, P.E. (2006) Nucleophilic Catalysis of Hydrazone Formation and Transimination: Implications for Dynamic Covalent Chemistry. *J. Am. Chem. Soc.*, **128**, 15602–15603.
[7] Aries, R. (1976) Hydrazone derivatives of rifamycin. Fr. Demande, 2,311,017, France.
[8] Saucic, A., Nitelea, I., Paunescu, E. *et al*. (1977) Rifamycins. Ger. Offen., 2,728,869, West Germany.
[9] Sharma, R., Saxena, D., Dwivedi, A.K. and Misra, A. (2001) Inhalable microparticles containing drug combinations to target alveolar macrophages for treatment of pulmonary tuberculosis. *Pharm. Res.*, **18**, 1405–1410.
[10] Singh, S., Mariappan, T.T., Sharda, N. *et al*. (2000) The Reason for an Increase in Decomposition of Rifampicin in the Presence of Isoniazid under Acid Conditions. *Pharm. Pharmacol. Commun.*, **6**, 405–410.
[11] Mariappan, T.T., Jindal, K.C. and Singh, S. (2004) Overestimation of rifampicin during colorimetric analysis of anti-tuberculosis products containing isoniazid due to formation of isonicotinyl hydrazone. *J. Pharm. Biomed. Anal.*, **36**, 905–908.
[12] Singh, S., Mariappan, T.T., Shankar, R. *et al*. (2001) A critical review of the probable reasons for the poor variable bioavailability of rifampicin from anti-tubercular fixed-dose combination (FDC) products, and the likely solutions to the problem. *Int. J. Pharm.*, **228**, 5–17.
[13] Singh, H., Bhandari, R. and Kaur, I.P. (2013) Encapsulation of Rifampicin in a solid lipid nanoparticulate system to limit its degradation and interaction with Isoniazid at acidic pH. *Int. J. Pharm.*, **446**, 106–111.
[14] Wang, H.F., Wang, R., O'Gorman, M. *et al.* (2013) Bioequivalence of fixed-dose combination Myrin(R)-P Forte and reference drugs in loose combination. *Int. J. Tuberc. Lung Dis.*, **17**, 1596–1601.
[15] Xu, J., Jin, H., Zhu, H. *et al*. (2013) Oral bioavailability of rifampicin, isoniazid, ethambutol, and pyrazinamide in a 4-drug fixed-dose combination compared with the separate formulations in healthy Chinese male volunteers. *Clin. Ther.*, **35**, 161–168.
[16] Milan-Segovia, R.C., Dominguez-Ramirez, A.M., Jung-Cook, H. *et al.* (2010) Relative bioavailability of rifampicin in a three-drug fixed-dose combination formulation. *Int. J. Tuberc. Lung Dis.*, **14**, 1454–1460.
[17] Pillai, G., Fourie, P.B., Padayatchi, N. *et al.* (1999) Recent bioequivalence studies on fixed-dose combination anti-tuberculosis drug formulations available on the global market. *Int. J. Tuberc. Lung Dis.*, **3**, S309–S316; discussion S317–S321.
[18] Alsultan, A. and Peloquin, C.A. (2014) Therapeutic drug monitoring in the treatment of tuberculosis: an update. *Drugs*, **74**, 839–854.
[19] Zumla, A., Nahid, P. and Cole, S.T. (2013) Advances in the development of new tuberculosis drugs and treatment regimens. *Nat. Rev. Drug Discov.*, **12**, 388–404.
[20] Zumla, A.I., Gillespie, S.H., Hoelscher, M. et al. (2014) New antituberculosis drugs, regimens, and adjunct therapies: needs, advances, and future prospects. *Lancet Infect. Dis.*, **14**, 327–340.
[21] Ziganshina, L.E., Titarenko, A.F. and Davies, G.R (2013) Fluoroquinolones for treating tuberculosis (presumed drug-sensitive). *Cochrane Database Syst. Rev.* **6** (Art. No.: CD004795). doi: 10.1002/14651858.CD004795.pub4.
[22] Jawahar, M.S., Banurekha, V.V., Paramasivan, C.N. *et al*. (2013) Randomized clinical trial of thrice-weekly 4-month moxifloxacin or gatifloxacin containing regimens in the treatment of new sputum positive pulmonary tuberculosis patients. *PLoS One*, **8**, e67030.
[23] Merle, C.S., Fielding, K., Sow, O.B. *et al*. (2014) A four-month gatifloxacin-containing regimen for treating tuberculosis. *New Engl. J. Med.*, **371**, 1588–1598.
[24] Warner, D.F. and Mizrahi, V. (2014) Shortening Treatment for Tuberculosis – Back to Basics. *New Engl. J. Med.*, **371**, 1642–1643.
[25] Dartois, V. and Barry, C.E. (2010) Clinical pharmacology and lesion penetrating properties of second- and third-line antituberculous agents used in the management of multidrug-resistant (MDR) and extensively-drug resistant (XDR) tuberculosis. *Curr. Clin. Pharmacol.*, **5**, 96–114.
[26] Dartois, V. (2014) The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. *Nat. Rev. Microbiol.*, **12**, 159–167.
[27] Jindani, A., Harrison, T.S., Nunn, A.J. *et al*. (2014) High-Dose Rifapentine with Moxifloxacin for Pulmonary Tuberculosis. *New Engl. J. Med.*, **371**, 1599–1608.

[28] Gillespie, S.H., Crook, A.M., McHugh, T.D. *et al*. (2014) Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis. *New Engl. J. Med.*, **371**, 1577–1587.
[29] Diacon, A.H., Dawson, R., von Groote-Bidlingmaier, F. *et al.* (2012) 14-day bactericidal activity of PA-824, bedaquiline, pyrazinamide, and moxifloxacin combinations: a randomised trial. *Lancet*, **380**, 986–993.
[30] Lienhardt, C., Raviglione, M., Spigelman, M. *et al.* (2012) New Drugs for the Treatment of Tuberculosis: Needs, Challenges, Promise, and Prospects for the Future. *J. Infect. Dis.*, **205**, S241–S249.
[31] Hickey, A.J., Misra, A. and Fourie, P.B. (2013) Dry powder antibiotic aerosol product development: inhaled therapy for tuberculosis. *J. Pharm. Sci.*, **102**, 3900–3907.
[32] Misra, A., Hickey, A.J., Rossi, C. *et al.* (2011) Inhaled drug therapy for treatment of tuberculosis. *Tuberculosis (Edinburgh)*, **91**, 71–81.
[33] Cockcroft, D.W. (1999) Pharmacologic therapy for asthma: overview and historical perspective. *J. Clin. Pharmacol.*, **39**, 216–222.
[34] Yadav, A.B., Singh, A.K., Verma, R.K. *et al.* (2011) The devil's advocacy: when and why inhaled therapies for tuberculosis may not work. *Tuberculosis (Edinburgh)*, **91**, 65–66.
[35] Fenaroli, F., Westmoreland, D., Benjaminsen, J. *et al*. (2014) Nanoparticles as drug delivery system against tuberculosis in zebrafish embryos: direct visualization and treatment. *ACS Nano*, **8**, 7014–7026.
[36] O'Hara, P. and Hickey, A.J. (2000) Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: manufacture and characterization. *Pharm. Res.*, **17**, 955–961.
[37] Vyas, S.P., Kannan, M.E., Jain, S. *et al*. (2004) Design of liposomal aerosols for improved delivery of rifampicin to alveolar macrophages. *Int. J. Pharm.*, **269**, 37–49.
[38] Coowanitwong, I., Arya, V., Kulvanich, P. and Hochhaus, G. (2008) Slow release formulations of inhaled rifampin. *AAPS J.*, **10**, 342–348.
[39] Mizoe, T., Ozeki, T. and Okada, H. (2008) Application of a four-fluid nozzle spray drier to prepare inhalable rifampicin-containing mannitol microparticles. *AAPS PharmSciTech.*, **9**, 755–761.
[40] Patomchaiviwat, V., Paeratakul, O. and Kulvanich, P. (2008) Formation of inhalable rifampicin-poly(L-lactide) microparticles by supercritical anti-solvent process. *AAPS PharmSciTech.*, **9**, 1119–1129.
[41] Tewes, F., Brillault, J., Couet, W. and Olivier, J.C. (2008) Formulation of rifampicin-cyclodextrin complexes for lung nebulization. *J. Control. Release*, **129**, 93–99.
[42] Tomoda, K., Ohkoshi, T., Kawai, Y. *et al.* (2008) Preparation and properties of inhalable nanocomposite particles: effects of the temperature at a spray-dryer inlet upon the properties of particles. *Colloids Surf., B: Biointerfaces*, **61**, 138–144.
[43] Changsan, N., Chan, H.K., Separovic, F. and Srichana, T. (2009) Physicochemical characterization and stability of rifampicin liposome dry powder formulations for inhalation. *J. Pharm. Sci.*, **98**, 628–639.
[44] Doan, T.V. and Olivier, J.C. (2009) Preparation of rifampicin-loaded PLGA microspheres for lung delivery as aerosol by premix membrane homogenization. *Int. J. Pharm.*, **382**, 61–66.
[45] Ohashi, K., Kabasawa, T., Ozeki, T. and Okada, H. (2009) One-step preparation of rifampicin/poly(lactic-co-glycolic acid) nanoparticle-containing mannitol microspheres using a four-fluid nozzle spray drier for inhalation therapy of tuberculosis. *J. Control. Release*, **135**, 19–24.
[46] Sung, J.C., Padilla, D.J., Garcia-Contreras, L. *et al*. (2009) Formulation and pharmacokinetics of self-assembled rifampicin nanoparticle systems for pulmonary delivery. *Pharm. Res.*, **26**, 1847–1855.
[47] Hirota, K., Hasegawa, T., Nakajima, T. *et al*. (2010) Delivery of rifampicin-PLGA microspheres into alveolar macrophages is promising for treatment of tuberculosis. *J. Control. Release*, **142**, 339–346.
[48] Doan, T.V., Couet, W. and Olivier, J.C. (2011) Formulation and in vitro characterization of inhalable rifampicin-loaded PLGA microspheres for sustained lung delivery. *Int. J. Pharm.*, **414**, 112–117.
[49] Son, Y.J. and McConville, J.T. (2011) A new respirable form of rifampicin. *Eur. J. Pharm. Biopharm.*, **78**, 366–376.
[50] Son, Y.J. and McConville, J.T. (2012) Preparation of sustained release rifampicin microparticles for inhalation. *J. Pharm. Pharmacol.*, **64**, 1291–1302.

[51] Hirota, K., Kawamoto, T., Nakajima, T. *et al*. (2013) Distribution and deposition of respirable PLGA microspheres in lung alveoli. *Colloids Surf., B: Biointerfaces*, **105**, 92–97.
[52] Singh, C., Bhatt, T.D., Gill, M.S. and Suresh, S. (2014) Novel rifampicin-phospholipid complex for tubercular therapy: synthesis, physicochemical characterization and in-vivo evaluation. *Int. J. Pharm.*, **460**, 220–227.
[53] Maretti, E., Rossi, T., Bondi, M. *et al.* (2014) Inhaled solid lipid microparticles to target alveolar macrophages for tuberculosis. *Int. J. Pharm.*, **462**, 74–82.
[54] Parikh, R., Patel, L. and Dalwadi, S. (2014) Microparticles of rifampicin: comparison of pulmonary route with oral route for drug uptake by alveolar macrophages, phagocytosis activity and toxicity study in albino rats. *Drug Deliv.*, **21**, 406–411.
[55] Vadakkan, M.V., Annapoorna, K., Sivakumar, K.C. *et al*. (2013) Dry powder cationic lipopolymeric nanomicelle inhalation for targeted delivery of antitubercular drug to alveolar macrophage. *Int. J. Nanomed.*, **8**, 2871–2885.
[56] Kumar, P.V., Asthana, A., Dutta, T. and Jain, N.K. (2006) Intracellular macrophage uptake of rifampicin loaded mannosylated dendrimers. *J. Drug Targeting*, **14**, 546–556.
[57] Chanwong, S., Maneekarn, N., Makonkawkeyoon, L. and Makonkawkeyoon, S. (2007) Intracellular growth and drug susceptibility of Mycobacterium tuberculosis in macrophages. *Tuberculosis (Edinburgh)*, **87**, 130–133.
[58] Jayaram, R., Gaonkar, S., Kaur, P. *et al*. (2003) Pharmacokinetics-pharmacodynamics of rifampin in an aerosol infection model of tuberculosis. *Antimicrob. Agents Chemother.*, **47**, 2118–2124.
[59] Mitchison, D. (2000) Role of individual drugs in the chemotherapy of tuberculosis. *Int. J. Tuberc. Lung Dis.*, **4**, 796–806.
[60] Rojanarat, W., Changsan, N., Tawithong, E. *et al.* (2011) Isoniazid proliposome powders for inhalation-preparation, characterization and cell culture studies. *Int. J. Mol. Sci.*, **12**, 4414–4434.
[61] Zhou, H., Lengsfeld, C., Claffey, D.J. *et al*. (2002) Hydrophobic ion pairing of isoniazid using a prodrug approach. *J. Pharm. Sci.*, **91**, 1502–1511.
[62] Pourshahab, P.S., Gilani, K., Moazeni, E. *et al.* (2011) Preparation and characterization of spray dried inhalable powders containing chitosan nanoparticles for pulmonary delivery of isoniazid. *J. Microencapsul.*, **28**, 605–613.
[63] Manca, M.L., Cassano, R., Valenti, D. *et al.* (2013) Isoniazid-gelatin conjugate microparticles containing rifampicin for the treatment of tuberculosis. *J. Pharm. Pharmacol.*, **65**, 1302–1311.
[64] Zhou, H., Zhang, Y., Biggs, D.L. *et al.* (2005) Microparticle-based lung delivery of INH decreases INH metabolism and targets alveolar macrophages. *J. Control. Release*, **107**, 288–299.
[65] Parikh, R. and Dalwadi, E. (2014) Preparation and characterization of controlled release poly-ε-caprolactone microparticles of isoniazid for drug delivery through pulmonary route. *Powder Technol.*, **264**, 158–165.
[66] Rojanarat, W., Nakpheng, T., Thawithong, E. *et al.* (2012) Inhaled pyrazinamide proliposome for targeting alveolar macrophages. *Drug Deliv.*, **19**, 334–345.
[67] Pham, D.D., Fattal, E., Ghermani, N. *et al*. (2013) Formulation of pyrazinamide-loaded large porous particles for the pulmonary route: avoiding crystal growth using excipients. *Int. J. Pharm.*, **454**, 668–677.
[68] Mitchison, D. and Davies, G. (2012) The chemotherapy of tuberculosis: past, present and future. *Int. J. Tuberc. Lung Dis.*, **16**, 724–732.
[69] Tsapis, N., Bennett, D., O'Driscoll, K. *et al.* (2003) Direct lung delivery of para-aminosalicylic acid by aerosol particles. *Tuberculosis (Edinburgh)*, **83**, 379–385
[70] Saifullah, B., Arulselvan, P., El Zowalaty, M.E. *et al*. (2014) Development of a highly biocompatible anti-tuberculosis nanodelivery formulation based on para-amino salicylic acid–zinc layered hydroxide nanocomposites. *Scientific World J.,* Article 401460.
[71] Shnittser, M.S. and Fai, T.I. (1959) [Experience with inhalation-tracheal administration of streptomycin in pulmonary tuberculosis therapy]. *Probl. Tuberk.*, **37**, 49–52.
[72] Rikimaru, T., Tanaka, Y., Higuchi, E. *et al.* (1992) [Evaluation of aerosol therapy of streptomycin for tracheobronchial and pulmonary tuberculosis]. *Kansenshogaku Zasshi*, **66**, 206–211.
[73] Sacks, L.V., Pendle, S., Orlovic, D. *et al.* (2001) Adjunctive salvage therapy with inhaled aminoglycosides for patients with persistent smear-positive pulmonary tuberculosis. *Clin. Infect. Dis.*, **32**, 44–49.

[74] Justo, O.R. and Moraes, A.M. (2005) Kanamycin incorporation in lipid vesicles prepared by ethanol injection designed for tuberculosis treatment. *J. Pharm. Pharmacol.*, **57**, 23–30.
[75] Olivier, K.N., Shaw, P.A., Glaser, T.S. *et al*. (2014) Inhaled amikacin for treatment of refractory pulmonary nontuberculous mycobacterial disease. *Ann. Am. Thorac. Soc.*, **11**, 30–35.
[76] Rose, S.J., Neville, M.E., Gupta, R. and Bermudez, L.E (2014) Delivery of Aerosolized Liposomal Amikacin as a Novel Approach for the Treatment of Nontuberculous Mycobacteria in an Experimental Model of Pulmonary Infection. *PLoS One*, **9**, e108703.
[77] Duan, J., Vogt, F.G., Li, X. *et al.* (2013) Design, characterization, and aerosolization of organic solution advanced spray-dried moxifloxacin and ofloxacin dipalmitoylphosphatidylcholine (DPPC) microparticulate/nanoparticulate powders for pulmonary inhalation aerosol delivery. *Int. J. Nanomed.*, **8**, 3489–3505.
[78] Ventura, C.A., Tommasini, S., Crupi, E. *et al.* (2008) Chitosan microspheres for intrapulmonary administration of moxifloxacin: interaction with biomembrane models and in vitro permeation studies. *Eur. J. Pharm. Biopharm.*, **68**, 235–244.
[79] Park, J.H., Jin, H.E., Kim, D.D. *et al.* (2013) Chitosan microspheres as an alveolar macrophage delivery system of ofloxacin via pulmonary inhalation. *Int. J. Pharm.*, **441**, 562–569.
[80] Hwang, S.M., Kim, D.D., Chung, S.J. and Shim, C.K. (2008) Delivery of ofloxacin to the lung and alveolar macrophages via hyaluronan microspheres for the treatment of tuberculosis. *J. Control. Release*, **129**, 100–106.
[81] Rojanarat, W., Nakpheng, T., Thawithong, E. *et al.* (2012) Levofloxacin-proliposomes: opportunities for use in lung tuberculosis. *Pharmaceutics*, **4**, 385–412.
[82] Sung, J.C., Garcia-Contreras, L., Verberkmoes, J.L. *et al.* (2009) Dry powder nitroimidazopyran antibiotic PA-824 aerosol for inhalation. *Antimicrob. Agents Chemother.*, **53**, 1338–1343.
[83] Garcia-Contreras, L., Sung, J.C., Muttil, P. *et al.* (2010) Dry Powder PA-824 Aerosols for Treatment of Tuberculosis in Guinea Pigs. *Antimicrob. Agents Chemother.*, **54**, 1436–1442.
[84] Dharmadhikari, A.S., Kabadi, M., Gerety, B. *et al.* (2013) Phase I, single-dose, dose-escalating study of inhaled dry powder capreomycin: a new approach to therapy of drug-resistant tuberculosis. *Antimicrob. Agents Chemother.*, **57**, 2613–2619.
[85] Giovagnoli, S., Blasi, P., Schoubben, A. *et al.* (2007) Preparation of large porous biodegradable microspheres by using a simple double-emulsion method for capreomycin sulfate pulmonary delivery. *Int. J. Pharm.*, **333**, 103–111.
[86] Ricci, M., Giovagnoli, S., Blasi, P. *et al*. (2006) Development of liposomal capreomycin sulfate formulations: effects of formulation variables on peptide encapsulation. *Int. J. Pharm.*, **311**, 172–181.
[87] Wang, C. and Hickey, A.J. (2010) Isoxyl aerosols for tuberculosis treatment: preparation and characterization of particles. *AAPS PharmSciTech*, **11**, 538–549.
[88] Sharma, R., Saxena, D., Dwivedi, A.K. and Misra, A. (2001) Inhalable microparticles containing drug combinations to target alveolar macrophages for treatment of pulmonary tuberculosis. *Pharm. Res.*, **18**, 1405–1410.
[89] Muttil, P., Kaur, J., Kumar, K. *et al.* (2007) Inhalable microparticles containing large payload of anti-tuberculosis drugs. *Eur. J. Pharm. Sci.*, **32**, 140–150.
[90] Pandey, R., Sharma, A., Zahoor, A. *et al.* (2003) Poly (DL-lactide-co-glycolide) nanoparticle-based inhalable sustained drug delivery system for experimental tuberculosis. *J. Antimicrob. Chemother.*, **52**, 981–986.
[91] Pandey, R. and Khuller, G. (2005) Solid lipid particle-based inhalable sustained drug delivery system against experimental tuberculosis. *Tuberculosis (Edinburgh)*, **85**, 227–234.
[92] Zahoor, A., Sharma, S. and Khuller, G. (2005) Inhalable alginate nanoparticles as antitubercular drug carriers against experimental tuberculosis. *Int. J. Antimicrob. Agents*, **26**, 298–303.
[93] Chan, J.G.Y., Chan, H.-K., Prestidge, C.A. *et al.* (2013) A novel dry powder inhalable formulation incorporating three first-line anti-tubercular antibiotics. *Eur. J. Pharm. Biopharm.*, **83**, 285–292.
[94] Katiyar, S.K., Bihari, S. and Prakash, S. (2008) Low-dose inhaled versus standard dose oral form of anti-tubercular drugs: Concentrations in bronchial epithelial lining fluid, alveolar macrophage and serum. *J. Postgrad. Med.*, **54**, 245–246.
[95] Chimote, G. and Banerjee, R. (2009) Evaluation of antitubercular drug loaded surfactants as inhalable drug delivery systems for pulmonary tuberculosis. *J. Biomed. Mater. Res., Part A*, **89**, 281–292.

[96] Justo, O.R. and Moraes, A.M. (2003) Incorporation of antibiotics in liposomes designed for tuberculosis therapy by inhalation. *Drug Deliv.*, **10**, 201–207.
[97] Chan, J., Tyne, A., Pang, A. *et al.* (2014) A Rifapentine-Containing Inhaled Triple Antibiotic Formulation for Rapid Treatment of Tubercular Infection. *Pharm. Res.*, **31**, 1239–1253.
[98] Labro, M.T. (2000) Interference of antibacterial agents with phagocyte functions: immunomodulation or "immuno-fairy tales"? *Clin. Microbiol. Rev.*, **13**, 615–650.
[99] Gordon, S. (2003) Alternative activation of macrophages. *Nat. Rev. Immunol.*, **3**, 23–35.
[100] Rajaram, M.V., Brooks, M.N., Morris, J.D. *et al*. (2010) Mycobacterium tuberculosis activates human macrophage peroxisome proliferator-activated receptor gamma linking mannose receptor recognition to regulation of immune responses. *J. Immunol.*, **185**, 929–942.
[101] Andrade, M.R., Amaral, E.P., Ribeiro, S,C. *et al.* (2012) Pathogenic Mycobacterium bovis strains differ in their ability to modulate the proinflammatory activation phenotype of macrophages. *BMC Microbiol.*, **12**, 166.
[102] Sharma, R., Muttil, P., Yadav, A.B. *et al.* (2007) Uptake of inhalable microparticles affects defence responses of macrophages infected with Mycobacterium tuberculosis H37Ra. *J. Antimicrob. Chemother.*, **59**, 499–506.
[103] Lewis, D.J., Williams, T.C. and Beck, S.L. (2014) Foamy macrophage responses in the rat lung following exposure to inhaled pharmaceuticals: a simple, pragmatic approach for inhaled drug development. *J. Appl. Toxicol.*, **34**, 319–331.
[104] Amaral, L., Martins, M. and Viveiros, M. (2007) Enhanced killing of intracellular multidrug-resistant Mycobacterium tuberculosis by compounds that affect the activity of efflux pumps. *J. Antimicrob. Chemother.*, **59**, 1237–1246.
[105] Gupta, S., Tyagi, S., Almeida, D.V. *et al*. (2013) Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an efflux inhibitor. *Am. J. Respir. Crit. Care Med.*, **188**, 600–607.
[106] Gupta, S., Cohen, K.A., Winglee, K. *et al*. (2014) Efflux inhibition with verapamil potentiates bedaquiline in Mycobacterium tuberculosis. *Antimicrob. Agents Chemother.*, **58**, 574–576.
[107] Elkington, P., Shiomi, T., Breen, R. *et al.* (2011) MMP-1 drives immunopathology in human tuberculosis and transgenic mice. *J. Clin. Invest.*, **121**, 1827–1833.
[108] Adams, K.N., Szumowski, J.D. and Ramakrishnan, L. (2014) Verapamil, and its metabolite norverapamil, inhibit macrophage-induced, bacterial efflux pump-mediated tolerance to multiple anti-tubercular drugs. *J. Infect. Dis.*, **210**, 456–466.
[109] Singh, K., Kumar, M., Pavadai, E. *et al.* (2014) Synthesis of new verapamil analogues and their evaluation in combination with rifampicin against Mycobacterium tuberculosis and molecular docking studies in the binding site of efflux protein Rv1258c. *Bioorg. Med. Chem. Lett.*, **24**, 2985–2990.
[110] Schmalstieg, A.M., Srivastava, S., Belkaya, S. *et al*. (2012) The antibiotic resistance arrow of time: efflux pump induction is a general first step in the evolution of mycobacterial drug resistance. *Antimicrob. Agents Chemother.*, **56**, 4806–4815.
[111] Rahn, K.H., Mooy, J., Bohm, R. and van der Vet, A. (1985) Reduction of bioavailability of verapamil by rifampin. *New Engl. J. Med.*, **312**, 920–921.
[112] Amaral, L., Kristiansen, J.E., Abebe, L.S. and Millett, W. (1996) Inhibition of the respiration of multi-drug resistant clinical isolates of Mycobacterium tuberculosis by thioridazine: potential use for initial therapy of freshly diagnosed tuberculosis. *J. Antimicrob. Chemother.*, **38**, 1049–1053.
[113] Bettencourt, M.V., Bosne-David, S. and Amaral, L. (2000) Comparative in vitro activity of phenothiazines against multidrug-resistant Mycobacterium tuberculosis. *Int. J. Antimicrob. Agents*, **16**, 69–71.
[114] Dutta, N.K., Pinn, M.L. and Karakousis, P.C. (2014) Sterilizing activity of thioridazine in combination with the first-line regimen against acute murine tuberculosis. *Antimicrob. Agents Chemother.*, **58**, 5567–5569.
[115] Singh, A. and Sharma, S. (2014) Chemotherapeutic efficacy of thioridazine as an adjunct drug in a murine model of latent tuberculosis. *Tuberculosis (Edinburgh)*, **94**, 695–700.
[116] Udwadia, Z.F., Sen, T. and Pinto, L.M. (2011) Safety and efficacy of thioridazine as salvage therapy in Indian patients with XDR-TB. *Recent Pat. Antiinfect. Drug Discov.*, **6**, 88–91.

[117] Abbate, E., Vescovo, M., Natiello, M. *et al.* (2012) Successful alternative treatment of extensively drug-resistant tuberculosis in Argentina with a combination of linezolid, moxifloxacin and thioridazine. *J. Antimicrob. Chemother.*, **67**, 473–477.
[118] Huang, T.S., Kunin, C.M., Wang, H.M. *et al*. (2013) Inhibition of the Mycobacterium tuberculosis reserpine-sensitive efflux pump augments intracellular concentrations of ciprofloxacin and enhances susceptibility of some clinical isolates. *J. Formos. Med. Assoc.*, **112**, 789–794.
[119] Escribano, I., Rodriguez, J.C., Llorca, B. *et al*. (2007) Importance of the efflux pump systems in the resistance of Mycobacterium tuberculosis to fluoroquinolones and linezolid. *Chemotherapy (Basel)*, **53**, 397–401.
[120] Sharma, S., Kumar, M., Nargotra, A. *et al.* (2010) Piperine as an inhibitor of Rv1258c, a putative multidrug efflux pump of Mycobacterium tuberculosis. *J. Antimicrob. Chemother.*, **65**, 1694–1701.
[121] Crowle, A.J., Salfinger, M. and May, M.H. (1989) 1,25(OH)2-vitamin D3 synergizes with pyrazinamide to kill tubercle bacilli in cultured human macrophages. *Am. Rev. Respir. Dis.*, **139**, 549–552.
[122] Salamon, H., Bruiners, N., Lakehal, K. *et al*. (2014) Cutting edge: Vitamin D regulates lipid metabolism in Mycobacterium tuberculosis infection. *J. Immunol.*, **193**, 30–34.
[123] Xia, J., Shi, L., Zhao, L. and Xu, F. (2014) Impact of vitamin D supplementation on the outcome of tuberculosis treatment: a systematic review and meta-analysis of randomized controlled trials. *Chin. Med. J. (Engl)*, **127**, 3127–3134.
[124] Kearns, M.D., Alvarez, J.A., Seidel, N. and Tangpricha, V. (2015) Impact of Vitamin D on Infectious Disease. *Am. J. Med. Sci.*, **349**, 245–262.
[125] Lobato, L.S., Rosa, P.S., Ferreira Jda, S. *et al.* (2014) Statins increase rifampin mycobactericidal effect. *Antimicrob. Agents Chemother.*, **58**, 5766–5774.
[126] Singh, V., Jamwal, S., Jain, R. *et al*. (2012) Mycobacterium tuberculosis-driven targeted recalibration of macrophage lipid homeostasis promotes the foamy phenotype. *Cell Host Microbe*, **12**, 669–681.
[127] Gutierrez, M.G., Master, S.S., Singh, S.B. *et al.* (2004) Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell*, **119**, 753–766.
[128] Fratazzi, C., Arbeit, R.D., Carini, C. *et al.* (1999) Macrophage apoptosis in mycobacterial infections. *J. Leukoc. Biol.*, **66**, 763–764.
[129] Gupta, A., Misra, A. and Deretic, V. (2016) Targeted pulmonary delivery of inducers of host macrophage autophagy as a potential host-directed chemotherapy of tuberculosis. *Adv. Drug Deliv. Rev.* doi: 10.1016/j.addr.2016.01.016.
[130] Gupta, A., Pant, G., Mitra, K. *et al*. (2014) Inhalable particles containing rapamycin for induction of autophagy in macrophages infected with Mycobacterium tuberculosis. *Mol. Pharmacol.*, **11**, 1201–1207.
[131] Yadav, A.B. and Misra, A. (2007) Enhancement of apoptosis of THP-1 cells infected with Mycobacterium tuberculosis by inhalable microparticles and relevance to bactericidal activity. *Antimicrob. Agents Chemother.*, **51**, 3740–3742.
[132] Yadav, A.B., Muttil, P., Singh, A.K. *et al.* (2010) Microparticles induce variable levels of activation in macrophages infected with Mycobacterium tuberculosis. *Tuberculosis (Edinburgh)*, **90**, 188–196.
[133] Yadav, A.B., Sharma, R., Muttil, P. *et al*. (2009) Inhalable microparticles containing isoniazid and rifabutin target macrophages and 'stimulate the phagocyte' to achieve high efficacy. *Indian J. Exp. Biol.*, **47**, 469–474.
[134] O'Sullivan, M.P., O'Leary, S., Kelly, D.M. and Keane, J. (2007) A caspase-independent pathway mediates macrophage cell death in response to Mycobacterium tuberculosis infection. *Infect. Immun.*, **75**, 1984–1993.
[135] Pettis, R.J., Hall, I., Costa, D. and Hickey, A.J. (2000) Aerosol delivery of muramyl dipeptide to rodent lungs. *AAPS PharmSci*, **2**, E25.
[136] Wang, C., Muttil, P., Lu, D. *et al*. (2009) Screening for potential adjuvants administered by the pulmonary route for tuberculosis vaccines. *AAPS J.*, **11**, 139–147.

[137] Rosas-Taraco, A.G., Higgins, D.M., Sanchez-Campillo, J. *et al.* (2009) Intrapulmonary delivery of XCL1-targeting small interfering RNA in mice chronically infected with Mycobacterium tuberculosis. *Am. J. Respir. Cell Mol. Biol.*, **41**, 136–145.

[138] Rosas-Taraco, A.G., Higgins, D.M., Sanchez-Campillo, J. *et al.* (2011) Local pulmonary immunotherapy with siRNA targeting TGFbeta1 enhances antimicrobial capacity in Mycobacterium tuberculosis infected mice. *Tuberculosis (Edinburgh)*, **91**, 98–106.

[139] Kelly, C., Yadav, A.B., Lawlor, C. *et al.* (2014) Therapeutic Aerosol Bioengineering of siRNA for the Treatment of Inflammatory Lung Disease by TNFalpha Gene Silencing in Macrophages. *Mol. Pharmacol.*, **11**, 4270–4279.

[140] Timmins, G.S., Master, S., Rusnak, F. and Deretic, V. (2004) Requirements for nitric oxide generation from isoniazid activation in vitro and inhibition of mycobacterial respiration in vivo. *J. Bacteriol.*, **186**, 5427–5431.x

[141] Jung, J.Y., Madan-Lala, R., Georgieva, M. *et al.* (2013) The intracellular environment of human macrophages that produce nitric oxide promotes growth of mycobacteria. *Infect. Immun.*, **81**, 3198–3209.

[142] Verma, R.K., Singh, A.K., Mohan, M. *et al.* (2012) Inhalable microparticles containing nitric oxide donors: saying NO to intracellular Mycobacterium tuberculosis. *Mol. Pharmacol.*, **9**, 3183–3189.

[143] Singh, R., Hussain, S., Verma, R. and Sharma, P. (2013) Anti-mycobacterial screening of five Indian medicinal plants and partial purification of active extracts of Cassia sophera and Urtica dioica. *Asian Pac. J. Trop. Med.*, **6**, 366–371.

[144] Shigyo, K., Ocheretina, O., Merveille, Y.M. *et al.* (2013) Efficacy of nitazoxanide against clinical isolates of Mycobacterium tuberculosis. *Antimicrob. Agents Chemother.*, **57**, 2834–2837.

[145] Lam, K.K., Zheng, X., Forestieri, R. *et al*. (2012) Nitazoxanide stimulates autophagy and inhibits mTORC1 signaling and intracellular proliferation of Mycobacterium tuberculosis. *PLoS Pathog.*, **8**, e1002691.

[146] Chan, J.G., Tyne, A.S., Pang, A. *et al*. (2014) A rifapentine-containing inhaled triple antibiotic formulation for rapid treatment of tubercular infection. *Pharm. Res.*, **31**, 1239–1253.

[147] Chan, J.G., Duke, C.C., Ong, H.X. *et al*. (2014) A novel inhalable form of rifapentine. *J. Pharm. Sci.*, **103**, 1411–1421.

[148] de Jesús Valle, M.J., Uranga, N.S., López, F.G. *et al*. (2007) Disposition of linezolid in the isolated rat lung after systemic and pulmonary drug delivery. *J. Antimicrob. Chemother.*, **60**, 1074–1079.

[149] Heuking, S., Rothen-Rutishauser, B., Raemy, D.O. *et al*. (2013) Fate of TLR-1/TLR-2 agonist functionalised pDNA nanoparticles upon deposition at the human bronchial epithelium in vitro. *J. Nanobiotechnol.*, **11**, 29.

[150] Ahmad, Z., Sharma, S. and Khuller, G.K. (2005) Inhalable alginate nanoparticles as antitubercular drug carriers against experimental tuberculosis. *Int. J. Antimicrob. Agents*, **26**, 298–303.

[151] Yadav, A.B., Sharma, R., Muttil, P. *et al.* (2009) Inhalable Microparticles Containing Isoniazid and Rifabutin Target Macrophages and "Stimulate the Phagocyte" to Achieve High Efficacy. *Indian J. Exp. Biol.*, **47**, 469–474.

[152] Yadav, A.B., Muttil, P., Singh, A.K. *et al*. (2010) Microparticles induce variable levels of activation in macrophages infected with *Mycobacterium tuberculosis*. *Tuberculosis (Edinburgh)*, **90**, 188–196.

[153] Verma, R.K., Singh, A.K., Mohan, M. *et al.* (2011) Inhaled therapies for tuberculosis and the relevance of activation of lung macrophages by particulate drug-delivery systems. *Ther. Deliv.*, **2**, 753–768.

[154] Sadeghi, S. and Karimi, E. (2006) Spectrophotometric determination of rifampicin through chelate formation and charge transfer complexation in pharmaceutical preparation and biological fluids. *Chem. Pharm. Bull. (Tokyo)*, **54**, 1107–1112.

[155] Polk, R.E. (1989) Drug-drug interactions with ciprofloxacin and other fluoroquinolones. *Am. J. Med.*, **87**, 76S–81S.

[156] Stutchbury, T.K., Vine, K.L., Locke, J.M. *et al*. (2011) Preclinical evaluation of novel, all-in-one formulations of 5-fluorouracil and folinic acid with reduced toxicity profiles. *Anti-Cancer Drugs*, **22**, 24–34.

[157] Teixeira, M.G., de Assis, J.V., Soares, C.G. *et al*. (2014) Theoretical and experimental study of inclusion complexes formed by isoniazid and modified beta-cyclodextrins: 1H NMR structural determination and antibacterial activity evaluation. *J. Phys. Chem. B*, **118**, 81–93.
[158] Verma, R.K., Germishuizen, W.A., Motheo, M.P. *et al*. (2013) Inhaled microparticles containing clofazimine are efficacious in treatment of experimental tuberculosis in mice. *Antimicrob. Agents Chemother.*, **57**, 1050–1052.
[159] Hettiarachchi, G., Nguyen, D., Wu, J. *et al*. (2010) Toxicology and drug delivery by cucurbit[n] uril type molecular containers. *PLoS One*, **5**, e10514.
[160] Patil, J.S. and Suresh, S. (2009) Physicochemical Characterization, in vitro Release and Permeation Studies of Respirable Rifampicin-Cyclodextrin Inclusion Complexes. *Indian. J. Pharm. Sci.*, **71**, 638–643.
[161] Reverchon, E., De Marco, I. and Della Porta, G. (2002) Rifampicin microparticles production by supercritical antisolvent precipitation. *Int. J. Pharm.*, **243**, 83–91.
[162] Hu, D., Liu, L., Chen, W. *et al.* (2012) A novel preparation method for 5-aminosalicylic acid loaded eudragit s100 nanoparticles. *Int. J. Mol. Sci.*, **13**, 6454–6468.
[163] Ghatnur, S.M., Sonale, R.S., Balaraman, M. and Kadimi, U.S. (2012) Engineering liposomes of leaf extract of seabuckthorn (SBT) by supercritical carbon dioxide (SCCO2)-mediated process. *J. Liposome Res.*, **22**, 215–223.
[164] Bhutani, H., Singh, S., Jindal, K.C. and Chakraborti, A.K. (2005) Mechanistic explanation to the catalysis by pyrazinamide and ethambutol of reaction between rifampicin and isoniazid in anti-TB FDCs. *J. Pharm. Biomed. Anal.*, **39**, 892–899.
[165] Verma, R.K., Kaur, J., Kumar, K. *et al*. (2008) Intracellular time course, pharmacokinetics, and biodistribution of isoniazid and rifabutin following pulmonary delivery of inhalable microparticles to mice. *Antimicrob. Agents Chemother.*, **52**, 3195–3201.
[166] Provost, C., Kinget, R. and Herbots, H. (1981) The influence of emulsifiers on drug diffusion from o/w creams. *Pharm. Weekbl. Sci.*, **3**, 248–251.
[167] Clearie, K.L., Williamson, P.A., Vaidyanathan, S. *et al*. (2010) Systemic bioavailability of hydrofluoroalkane (HFA) formulations of fluticasone/salmeterol in healthy volunteers via pMDI alone and spacer. *Br. J. Clin. Pharmacol.*, **69**, 637–644.
[168] Verma, R.K., Mukker, J.K., Singh, R.S. *et al*. (2012) Partial biodistribution and pharmacokinetics of isoniazid and rifabutin following pulmonary delivery of inhalable microparticles to rhesus macaques. *Mol. Pharmacol.*, **9**, 1011–1016.
[169] Hourcade-Potelleret, F., Lemenuel-Diot, A., McIntyre, C. *et al*. (2014) Use of a population pharmacokinetic approach for the clinical development of a fixed-dose subcutaneous formulation of trastuzumab. *CPT Pharmacometrics Syst. Pharmacol.*, **3**, e87.
[170] Battaglia, R., Pianezzola, E., Salgarollo, G. *et al*. (1990) Absorption, disposition and preliminary metabolic pathway of 14C-rifabutin in animals and man. *J. Antimicrob. Chemother.*, **26**, 813–822.
[171] Kaur, J., Muttil, P., Verma, R.K. *et al*. (2008) A hand-held apparatus for "nose-only" exposure of mice to inhalable microparticles as a dry powder inhalation targeting lung and airway macrophages. *Eur. J. Pharm. Sci.*, **34**, 56–65.
[172] Hoppentocht, M., Hoste, C., Hagedoorn, P. *et al.* (2014) In vitro evaluation of the DP-4M PennCentury insufflator. *Eur. J. Pharm. Biopharm.*, **88**, 153–159.

12

Ion Pairing for Controlling Drug Delivery

Stefano Giovagnoli, Aurélie Schoubben and Carlo Rossi

Department of Pharmaceutical Sciences, University of Perugia, Perugia, Italy

12.1 Introduction

In recent years, different strategies have been employed to tune molecule properties so as to meet well-established therapeutic needs. Among all these strategies, chemical modification provides wide possibilities that have led to successful development of smart therapeutic approaches (e.g., the pro-drug approach). In this way, drug molecules can be modified, theoretically modulating either pharmacodynamics or pharmacokinetics in a target-defined fashion with virtually no limitations. Nevertheless, a modified drug represents a novel entity under both chemical and regulatory points of view, and this poses major constraints to the bench-to-market fast track.

Albeit possessing some common features with chemical modification, physical association of drugs with selected ionic ligands may be, in many ways, more advantageous as, supposedly, it does not alter a drug chemical structure, thereby increasing the chance of expedited approval by the regulatory agencies.

Nowadays, ion pairing is increasingly employed to serve such purposes since, in some cases, ionic interaction is energetically favored and association occurs almost spontaneously.

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

12.2 Ion Pairing Definitions and Concepts

The term ion pairing is regarded as the "*association of oppositely charged ions in solution to form distinct chemical species*" [1]. Pairing strength depends on several factors accounting for physicochemical properties of the species involved in the interaction. Albeit ion pairs are characterized by long-range interactions, binding can be strong enough to allow the formation of stable chemical entities with different properties and behaviors compared with the parent compounds.

The history and evolution of the concept of ion pairing encompass nearly 100 years and is beyond the aim of this chapter.

What we know today about the association of charged species in solution relies on the Debye–Huckel [2] and Onsager [3] models as well as Bronsted's theory [4] that allowed the development of the theories of Bjerrum [5], Fuoss [6, 7] and many others.

Today, although some controversies and unresolved issues exist, ion pairs are generally accepted as being real species. Some criteria have been indicated as to when ionic interaction can lead to ion pair formation. Ion pairs form when two charged species approach below a cutoff distance R. The equilibrium distance r is established as being at $a<r<R$, where a is the so-called '*distance of closest approach*', which represents the repulsive barrier constraining further vicinity.

Long-range electrostatic forces obeying Coulomb's law of attraction are generally recognized as being responsible for the interaction.

The solvent has a double role in ion pairing. On the one side, solvent permittivity decreases the strength of long-distance attraction and, on the other side, solvent molecules can take part in ion pairing, producing three different situations: solvent-separated, solvent-shared and contact ion pairs. The three models are summarized in Figure 12.1.

Unarguably, among the three situations the contact ion pair is the one granting the formation of stronger interactions and therefore this type of ion pair has a higher chance to exist. The solvent-separated and solvent-shared are loose types of ion pairs that are more difficult to detect and to measure [8].

Such models, although generally applicable, do not represent tightly more complex situations, such as that of multivalent ion pairs. This is the case for large molecular assemblies,

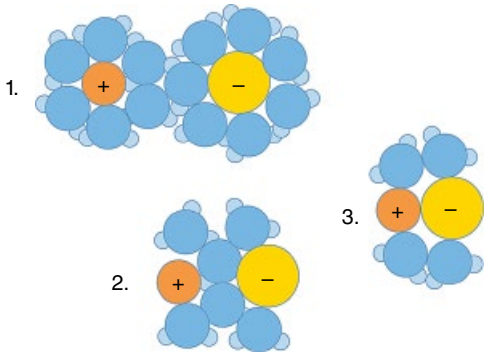


Figure 12.1 *Solvent contribution, represented by water molecules, to the formation of ion pairs: (1) solvent-separated ion pairs, (2) solvent-shared ion pairs and (3) contact ion pairs*

such as polyelectrolytes, and other large drug molecules. In these cases, ion pairing can generate more complex associations and secondary interactions in solution. In this regard, solvation plays a complicated role in determining the so-called counterion condensation of polymer chains in salt-free polyelectrolyte solutions [9]. Addition of multivalent salts can give rise to unexpected behaviors, such as like-charge attractive interactions, which are responsible for polyion condensation, as observed for DNA and other biomacromolecules in non-physiologic conditions [9, 10].

Generally, common nomenclature distinguishes ion pairs from complexes as the latter are defined when short-range coordination bonds occur. However, such a distinction [1] can be considered mainly semantic since neither the nature of the bond nor kinetic criteria can be applied to discern between ionic pairing and coordination. In fact, ionic interactions can be as strong as coordination bonds and, in certain conditions, kinetically favored [1]. Therefore, complexes and ion pairs may be regarded as special cases of the same phenomenon.

In this chapter, we will maintain the semantic distinction between complexes and ion pairs to distinguish among species resulting from binding with transition metals and other ionic compounds.

12.2.1 Ion Pairing as Physicochemical Tuning Tool

Accurate selection of ionic partners can be tremendously useful to modulate active molecule properties especially when such properties are unfavorable to therapeutic efficacy. In the last twenty years, works have proliferated in this area, applying ion pairing to a number of different molecules as well as to macromolecules [11]. A typical application of ion pairing as well as complexation is the modulation of a drug hydrophilic/hydrophobic balance (HHB). In this way, ion pairing can be used to produce partitioning of highly hydrophilic drugs, as in the case of leuprolide acetate [12] and the cephalosporins cefepime and cefpirome [13], in lipophilic environments, thus improving absorption through membranes. Nevertheless, the transmembrane transport process of ion pairs is not straightforward as it is sensitive to the nature of the vehicle medium. In fact, water-rich solvents can be highly structured into supramolecular assemblies producing ion–ion weak interactions with the ion pair that significantly influence transmembrane transport [14]. As is easily predictable, ion pairing enables not only hydrophilic drug partitioning in hydrophobic environments, but can be used to tune drug-dissolution profiles as well [15]. Such an effect is bound though to the hydrophobicity of the organic counterion used and the ionic strength of the solution.

The choice of the ionic partners should be based on the reciprocal affinity and the relative HHB. Popular counterions are picked among mild ionic surfactants, e.g. sodium dodecyl sulfate and octadecyl sulfate. These surfactants have been used to bind tacrine or *l*-phenylephrine [15] or biomolecules [16] such as subtilisin BPN' [17].

Biocompatibility is a crucial factor that needs to be accounted for in developing marketable ion pairs, which means that natural or semisynthetic compounds are preferable. Medium-to-long-chain fatty acids couple especially with peptides and can be employed as penetration enhancers of hydrophilic molecules, such as water-soluble amino acids through the skin [18] or other physiological barriers. In this regard, peptides can be ion paired with some organic acid derivatives, such as salicylate and 5-methoxysalicylate, to improve absorption in the organism or combined with inorganic anions to allow selective extraction [19].

Ion pairing has been correlated even with the innate capacity of arginine-rich cell-penetrating peptides to enter cells through a non-endocytotic pathway. This process is theoretically impeded by the extremely high Born free-energy barrier generated by the exposure of the charged molecule to the low-dielectric lipid layer. Evidence supports the mediating action of phospholipid/peptide ion pairing with subsequent charge neutralization [20]. This should allow the peptides to overcome the Born barrier and travel across the cell membrane.

In light of the aforementioned ability of arginine-rich peptides to penetrate cells, long-chain arginine esters have been used as counterions to bind plasmid DNA for cell transfection, showing reduced toxicity compared with other polycationic species [21].

It is evident that ion pairing of molecules of pharmaceutical interest can open many perspectives. This strategy could be of clinical relevance especially when considering the targeting of hydrophobic physiological environments using highly hydrophilic active compounds.

12.2.2 Metal Ion Complexation

Transition metals are classified as Lewis acids due to their electron-acceptor capacity. In fact, the availability of empty hybrid orbitals allows allocation of electron pairs from donor counterparts, such as amines, amides and/or carboxyl groups. This donor/acceptor interaction produces the coordination bond, which can be regarded as a result of a Lewis acid–base reaction.

The high aptitude to coordination of metals explains their ability to form complexes with a number of ions, as well as organic and inorganic ligands. The orientation of coordination bonds follows the spatial arrangement of hybrid orbitals that assume distinct and well-established geometries (Figure 12.2a).

As a result, transition metals can be chelated through so-called monodentate or polydentate links by organic molecules, especially those bearing multiple binding centers [e.g., ethylenediaminetetraacetic acid (EDTA)] (Figure 12.2b).

As anticipated, albeit experts in the field associate (conceptually) metal complexation to ion pairing [1], we will consider them distinct phenomena due to the theoretical different covalent nature of the coordination bond compared with that of the ionic bond. Energetically there is not much difference between the two kinds of bonds, as ionic interaction can be as strong as coordination and this is one of the reasons for the controversy in this distinction.

This also explains the random use of the term '*complex*' that is often associated to ion pairs as well. Transition metal coordination may be accounted as a mono- or multivalent ion pairing process according to the chelation ability of the ligand as depicted in Figure 12.2b.

Historically, metal complexes have been known to possess therapeutic relevance for centuries; however, the modern era of metal complexes started in 1909 with Erlich's arsphenamine, an organoarsenic compound used to treat syphilis [22].

Today, there are several metal complexes being used in therapy, such as those of Pt, which is currently employed in anticancer agents (i.e., cisplatin, carboplatin and oxaliplatin), and several others are under investigation [23]. Gold complexes like auranofin and myocrisin find application in rheumatoid arthritis treatment.

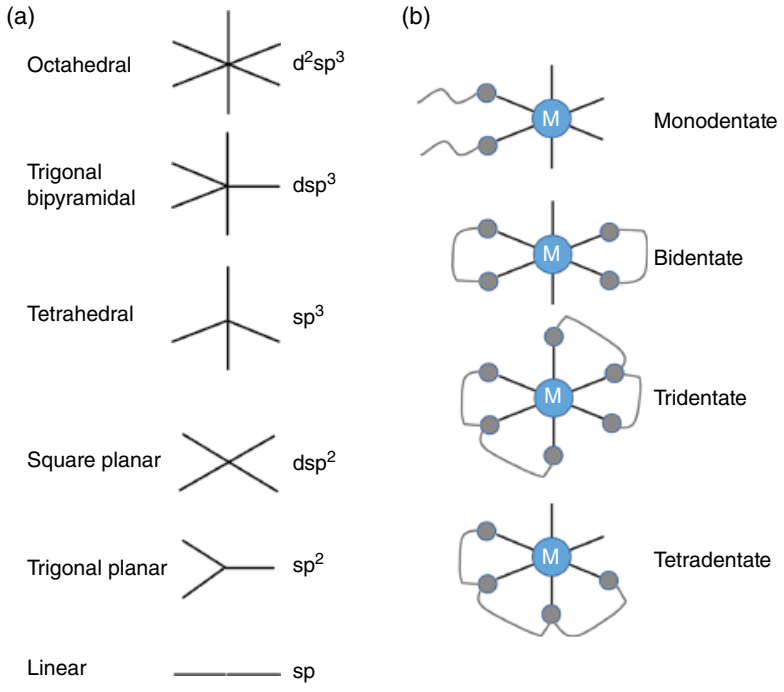


Figure 12.2 *(a) Examples of possible transition metal orbital hybridizations and (b) metal coordination chelates in complexes*

It is easy to understand why organometallic compounds find noticeable application, mainly as anticancer as well as antibiotic agents [24–26]. Most of the metals from the 4th to the 6th period have been combined with organic ligands. Some of them are of biological relevance, such as Cu, Zn, Ni, Co, Fe, as they are essential microelements in a cells lifecycle and in biological molecules (i.e., metalloenzymes, hemoglobin). Some others are heavy metals, such as Au, Pd, Re, Pb, Ru, Zr, Hg, Mo, Ag whose toxicity provides an innate antibacterial action. Nevertheless, in some cases genotoxicity and cancerogenicity have to be accounted as side effects.

These metals can coordinate not only small organic ligands but also large molecules of pharmaceutical interest and small drug molecules. In this case, metal binding can produce insoluble complexes as a result of the occupation by the metal of the polar groups previously engaged in water hydration. In this way, water-soluble molecules can be turned into more lipophilic complexes, resulting in HHB modulation. Some of these molecules can be large aminoglycoside and peptide antibiotics presenting N, O, S donor centers that can be engaged by the metal. Metal complexation has been successfully employed with a number of antibiotics and other drugs: ciprofloxacin and amoxicillin [27, 28], hydroxyquinoline [29, 30], cephalosporins [31], chloroquine [32], lansoprazole [33].

A comprehensive report on metal complexes is not the aim of this chapter, which is, rather, focused on the use of metal binding alongside ion pairing for the modification of drug properties. An overview of their use in drug delivery is provided hereafter along with ion pair applications.

12.2.3 Some Considerations on Ion Pair and Metal Complex Stability

To be classified as actual distinct species, ion pairs and complexes should be stable enough to be a recognizable kinetic entity. The formation of an ion pair in solution can be summarized by Equation (12.1) [1]:

$$C^{c+} + A^{a-} \leftrightarrow CA^{(c-a)+} \tag{12.1}$$

where C^{c+} and A^{a-} are the cation and the anion with charge c and a, respectively. A similar equation can be written for metal complexation by replacing the cation and anion with the metal and the ligand terms. The process can be quantified by the association constant K_A that is defined by the usual mass action law. Indicating the fraction of free ions and associated ions with α and $1-\alpha$, respectively, K_A assumes the expression {Equation (12.2)}.

$$K_A = \frac{(1-\alpha)c}{(\alpha c)^2} \tag{12.2}$$

where c is again the charge of the cation.

The fraction α can be measured by several methods. Nevertheless, due to the labile nature of some ion pairs, for which lifetimes as low as 1 ns have been reported [34], such determinations are not simple.

Equations (12.1) and (12.2) represent the formation of binary ion pairs, which is not the case for many multivalent compounds and complexes. In addition, solvent contribution is rather important as has already been pointed out previously. In this regard, the free energy of association at the equilibrium can be obtained from the van't Hoff's thermodynamic relation with K_A {Equation (12. 3)}.

$$\Delta G^0 = -RT \ln K_A^0 \tag{12.3}$$

where R and T are the well-known universal gas constant and absolute temperature. Evidence supports the hypothesis that solvation and desolvation phenomena influence considerably the ion pair equilibrium at the expense of the association enthalpy, which results often being positive owing to the energy required for ion desolvation. The consequence is that, a negative association free energy being required to stabilize the ion pair, ion pairing is, in many cases, an entropy-driven process. Of course, this condition is expected to occur at high solvation energies.

According to this consideration the standard enthalpy change upon ion pairing, as well as the recorded entropy values, can be used to establish the type of ion pair being formed; for example, solvent-separated or contact ion pairs (see Figure 12.1). This is because, beyond the interaction among ions, a complex interplay between the arrangement of solvent molecules on the ion pair and those being released during desolvation occurs, which determines the entropy and enthalpy energies according to Gibbs' law.

As already mentioned, ion-pair and complex stability is bound to the nature of partners; however, parameters such as temperature, pH and ionic strength, taken together, can determine the lifetime of ion pairs. Whether an ionic interaction or coordination bond occurs, stable pairing may depend not only on the reaction conditions but also on the preservation of such conditions over time. In fact, even apparently strong ion pairs and complexes can

undergo abrupt destabilization upon relatively unfavorable conditions, dictated by the presence of competing ligands or ions as well. Such phenomena are seldom predictable and, if conceivable, need to be evaluated. In this regard, ongoing debates on the fate of complexes and ion pairs once administered into the body highlight the need for insightful understanding of their stability when challenged with unfavorable and unpredictable interacting environments, such as cells and tissues. Therefore, determination of K_A in simulated biological environments alongside toxicity assessment are mandatory prior to transferring the use of such compounds to the clinic.

12.3 Ion Pairs, Complexes and Drug Delivery

The possibility to modulate drug properties by modifying the HHB can make the ion pairing and complexation approach a powerful strategy to improve therapeutic performances. Some work has been done in an attempt to pair drugs of interest, showing pharmacokinetic limitations due to physicochemical flaws, to proper cations (metals as well) or anions. Here we report the application of ion pairing and metal complexation to a number of different drug molecules sorted by administration route.

12.3.1 Oral Route

Ion pairing has been used in several studies to modulate active pharmaceutical ingredient permeability through the gastrointestinal tract, where the main mechanism of absorption is the transcellular route. To obtain a high oral bioavailability, drugs should possess a good balance between solubility and permeability. Highly water-soluble compounds are often poorly permeable through biological membranes. However, insoluble drugs are also problematic for oral administration. In a recent study, indomethacin was investigated for its ability to form ion pairs with arginine and lysine at different molar ratios [35]. Using this strategy, indomethacin log *P* progressively decreased from about 3 to 0.5 when 1:8 (drug:arginine/lysine) molar ratio was used. Besides the solubility improvement, permeability was significantly improved at 1:2 (drug:amino acid) molar ratio. These results indicated a possible intestinal absorption mediated by active carriers, while passive uptake should be less pronounced because of the lower log *P* [35]. The ion pairing approach has been mainly used to increase the apparent permeability of very soluble drugs except for the indomethacin case study. Different active pharmaceutical ingredients have been taken into consideration in the last decade. Amifostine is a polar cytoprotective compound used to protect normal tissues from chemotherapy and radiotherapy toxic effects. It is commonly administered by parenteral route to avoid its degradation and poor absorption in the gastrointestinal tract. Amifostine has been coupled with different organic acids (e.g., succinic acid, phthalic acid, benzoic acid) to evaluate their effect on the permeability profile [36, 37]. The data evidenced a significant increase of permeability and a bioavailability enhancement of 20 – 30 times and 10 times for succinic acid and phthalic acid, respectively, once administered in the rat duodenum. Therefore, ion paired amifostine could potentially be administered orally after an enteric coating, thereby avoiding the drawbacks of the parenteral route [36, 37]. Ion pairing was also studied to enhance the permeability of two different antiviral drugs, namely zanamivir heptyl ester and guanidino-oseltamivir [38, 39]. These papers

evidenced the importance not only of the optimization of molar ratio between the drug and the counterion but also of the binding constant. Here, 1-hydroxy-2-naphthoic acid (HNAP) was used as ion pairing agent and a high dissociation was observed with guanidino-oseltamivir impairing the approach employed. In fact, the hydrophobic ion pair should be stable in biological fluid and especially in the presence of competing ions present in the gastrointestinal tract (e.g., bile acids). Ion pairing was useful to enhance permeability of zanamivir heptyl ester in the rat jejunum [38]. Tetraheptylammonium bromide (THAB) or, alternatively, tetrabutylammonium iodide (TBAI) was used to increase oral bioavailability of alendronate. This drug, used in the treatment of osteoporosis and Paget's disease among others, is already available in tablets for oral administration but its bioavailability is lower than 1%. Having five pK_a values, ion pairing was investigated at different pH values and molar ratios [40]. Interestingly, the best molar ratio was 1:10 (drug:ion pairing agent) independently of the ion pairing agent used but the optimal pH conferring the highest hydrophobicity was 2.2 for THAB and 10.3 for TBAI [40]. These results enable attractive perspectives for the improvement of alendronate oral bioavailability. Sodium deoxycholate has been employed to produce ion pairs with insulin [41] and diltiazem hydrochloride [42]. A zinc chelate has been synthesized to improve gastrointestinal absorption of glimepiride, a third-generation hypoglycemic sulfonylurea for the treatment of non-insulin-dependent diabetes [43]. However, in this regard, as yet no updated development is available.

12.3.2 Transdermal/Dermal and Mucosal Route

The ion pairing approach can be useful to improve transdermal delivery by enhancing drug penetration through the *stratum corneum*. Even though hydrophobic ion pairing improves drug lipophilicity, it is necessary to accurately select the counterion to obtain the highest benefits in term of transport through the biological membranes. In fact, a high partition coefficient is not enough to grant transport through the *stratum corneum*, which is the product of both partition and diffusion. Commonly, ion pairs, being larger, show slower diffusion and transport through the skin [44]. Teriflunomide and lornoxicam were ion paired with different organic amines, enhancing their percutaneous absorption. The choice of the amine partner is based on the ability to warrant longer ion pair lifetime thus enhancing diffusion through the skin [45, 46]. The flux of salicylate, a hydrophilic ionic drug, was increased up to 11-fold when ion paired with alkylamines thanks to the significant increase of its solubility (137-fold) in isopropyl myristate carrier. The highest concentration gradient is therefore responsible for this effect and it was hypothesized that, once in the skin, the ion pair dissociates because of the aqueous microenvironment of the epidermis [47]. In another study on salicylate ion pairs, no significant effect on the *in vitro* penetration through the epidermis was observed, but an increased deposition of the drug in the skin was detected [48]. As stated before, the carrier is of fundamental importance to obtain permeation. This is the case of retinoic acid paired with a methyl or ethyl ester of different amino acids and incorporated in the internal phase of an oil-in-water microemulsion. Using this vehicle, the skin accumulation of the ion pairs was enhanced and the systemic effects were avoided [49].

Another example is a methotrexate/L-arginine ion pair for intranasal delivery useful to increase the drug partition coefficient and transport in the presence of propylene glycol [50].

12.3.3 Parenteral Route

Hydrophobic ion pairing has been investigated for parenteral delivery of peptides and proteins as well. These molecules suffer from several disadvantages such as low stability, short half-life, and, in particular, high aqueous solubility, which limits encapsulation in polymeric or lipid delivery systems. Major techniques for the preparation of polymeric micro-/nano-particulates are emulsion solvent diffusion-evaporation/extraction, coacervation, salting out, spray-drying, spray-congealing, and supercritical fluids-based methods. All these techniques share similar drawbacks in the case of peptides and proteins such as the well-known low encapsulation efficiency [51], high initial burst effect [51, 52], and degradation during manufacture or release [53, 54], for which, indeed, ion pairing can provide huge benefits. In this regard, melittin paired to sodium dodecyl sulfate [55] led to almost 100% encapsulation efficiency. A significant reduction of the initial burst release from poly(lactic-co-glycolic acid) (PLGA) carriers was obtained by ion pairing insulin with sodium deoxycholate [56], leuprolide with sodium oleate [57] and lyzozyme with dextran sulfate [58]. Ion pairing has been demonstrated to not alter drug activity, as in the case of insulin–lauryl sulfate complex [59]. Moreover, ion pairing is able to improve peptide or protein stability when in contact with organic solvents during encapsulation [55,60]. Ion pairing with oligosaccharide ester derivatives has been employed to tune release kinetics of peptide/proteins from particles as well [61]. The ion pairing approach is also very promising when applied to monoclonal antibodies. These large proteins are highly potent therapeutics characterized by short biological half-lives. Once ion paired, their encapsulation could guarantee the reduction of administration frequency by maintaining their structure integrity and activity [62]. Lastly, antibiotics, such as gentamicin, can be complexed to obtain derivatives that possess the same activity but an increased ability to cross biological membranes. This allows theorizing of their utilization in the treatment of intracellular infections [63].

12.3.4 The Pulmonary Route and Infectious Diseases

Using ion pairs and complexes as drug delivery systems to target the lungs seems to be a promising strategy to allow local accumulation of hydrophilic and certain ionizable lipophilic drugs. As remarked on above, in light of the possibility to turn such molecules into insoluble or low water-soluble compounds, this approach can prevent systemic absorption and subsequent biodistribution of the drug. The consequences of such an approach are a decrease in the potential long-term side effects as well as increased drug retention into the organ, which can result in a much lower allowable dosage and higher efficacy. This therapeutic strategy can be particularly useful to treat lung infections. In this regard, most of the molecules investigated for ion pairing and metal coordination are variably soluble antibiotic drugs owing to their chemical nature that allows easy pairing with metals and hydrophobic anions. Following the same approach, octreotide acetate has been hydrophobically ion paired to improve encapsulation in nanoparticle aggregates to be delivered to the lungs [64].

Yet, advances in this field are lagging as is rather evident by the limited literature available, and most works clog up at the pre-formulation stage.

12.3.4.1 The Special Case of Tuberculosis

The advantages listed above clearly suggest that ion pairing and complexation are compatible with application to the treatment of pulmonary infectious diseases, in particular tuberculosis (TB). Since TB infects hosts intracellularly and, in case of resistance, persistent bacteria seem to locate into low-permeable nearly anoxic lipophilic environments, development of penetrating compounds able to perfuse through membranes and the caseous medium in the tuberculi is desirable. Ion pairs and metal complexes may provide such properties and therefore allow administration and retention into the lungs of otherwise water-soluble antitubercular drugs (ATDs) that are prone to rapid systemic absorption. Dry powder inhalation of mixed drug complexes is practically feasible and it may provide huge benefits by wiping out the dramatic pharmacokinetic pitfalls of current TB therapy and allowing drug reappraisal [65]. In fact, one aspect of TB eradication failure comes from the impossibility to deliver exact ATD combinations at the infection site through conventional routes. Most literature in the field explores inhalable ATD loaded micro-/nano-particles [66–68]; however, this approach may not prevent massive drug absorption into the blood stream once particles have come in contact with lung fluids. On the other hand, we solicit the use of ion pairing or complexation for TB treatment not only for the potential eradication, but rather for the control of TB infection. In this regard, the first-line ATD isoniazid has been ion paired to improve transport across biological barriers [69] and embedded into microparticles to target alveolar macrophages [70]. We applied a similar approach to previously complexed second-line quinolones [71] and aminoglycoside ATDs that have been turned into insoluble Pd complexes, and tested for their activity against susceptible *M. tuberculosis* [72]. The obtained structures are reported in Figure 12.3.

Ofloxacin (Ofx), capreomycin (C) and kanamycin (K) Pd complexes, in particular, showed an extracellular activity comparable to that of the free drugs, as well as some superior intracellular efficacy (Figure 12.4).

The ofloxacin–Pd and capreomycin–Pd complexes were even microencasulated to produce inhalable biodegradable microparticles (Figure 12.5a, 12.5b) [73, 74]. Moreover, we also synthesized a novel capreomycin oleate ion pair that was micronized for possible lung delivery (Figure 12.5c, 12.5d) [75].

Capreomycin oleate is insoluble and showed an activity comparable to that of the parent drug. These systems are theoretically designed to enhance effective targeting of mycobacteria in cells and caseous tissue. Other ion pairing and complexation alternatives are currently under evaluation.

12.3.5 Toxicity Considerations

The limited number of publications in this field partly explains the lack of data addressing the toxicity of ion pairs and metal complexes. Toxicity of ion pairs and metal complexes is related to the properties of each molecular partner. However, pairing can change the way molecules interact with the environment, allowing access to sites otherwise inaccessible or enhancing interaction with certain molecular targets. Therefore, great attention should be paid to the assessment of toxic effects of such compounds.

As shown in Table 12.1 in the case of the capreomycin oleate ion pair [75], using GRAS (generally recognized as safe) materials as ionic partners is advantageous since it may grant to a higher extent preservation of a low toxicological profile.

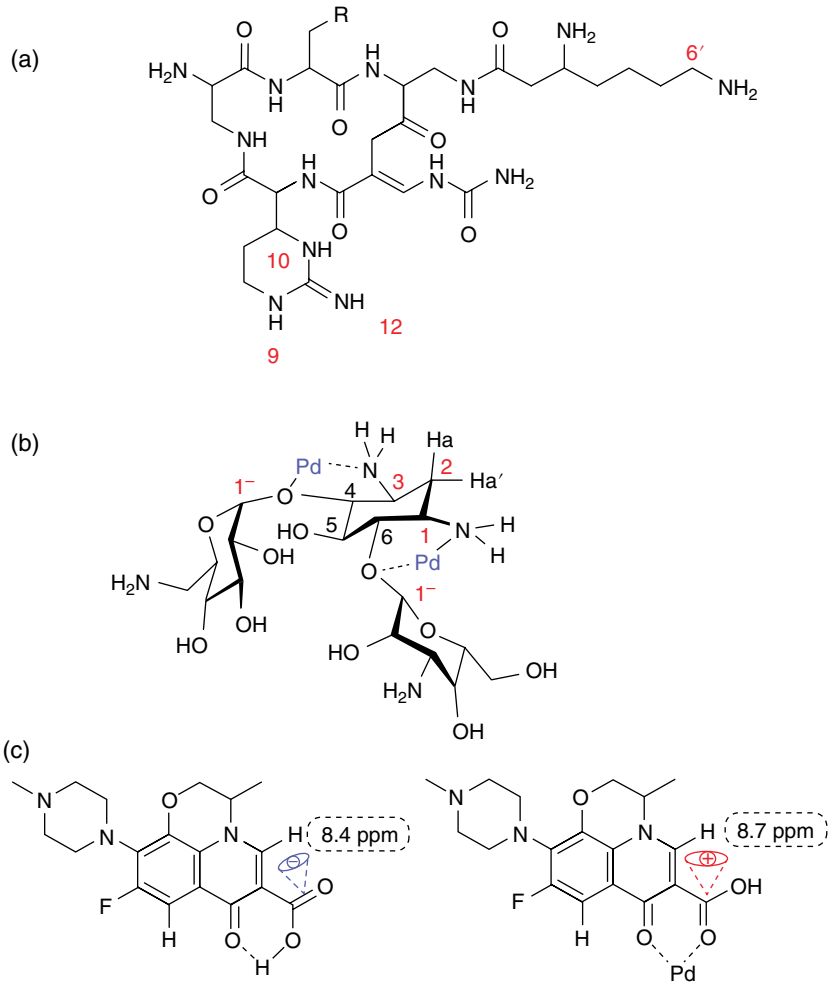


Figure 12.3 *Proposed structures and Pd chelation of (a) capreomycin, (b) kanamycin and (c) ofloxacin obtained by NMR measurements. The nuclei supposedly involved in the interaction with the metal are indicated (adapted with permission from Ref. [72]). (See insert for color representation of the figure.)*

Such an advantage is even more evident when looking at the different effects on chorioallantoic membrane of chicken embryos of the free drug and the ion pair that showed lower toxic effect with no abnormal vascularization and lesions, as in turn was observed for capreomycin alone (Figure 12.6).

Predictably, owing to its surface activity, sodium oleate is even more toxic than the drug itself and this enforces the hypothesis that ion pairing can in some cases reduce the intrinsic toxic potential of non-biocompatible substances. Of course, in many other situations these effects may not be expected.

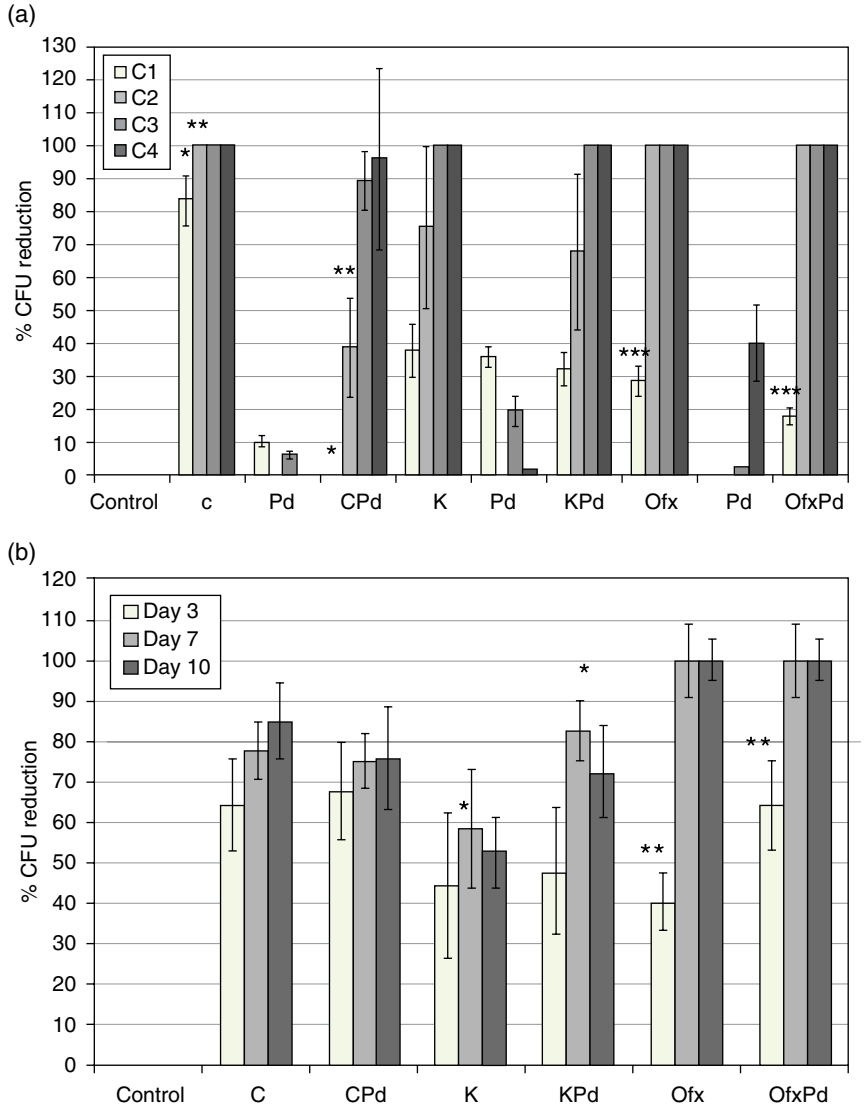


Figure 12.4 *a) Extracellular activity (C1–C4 = 0.25–10 μg/mL) and b) intracellular activity in infected THP-1 cells, a human myelomonocytic cell line with macrophage-like activity, (2–10 μg/mL) of capreomycin–Pd, ofloxacin–Pd and kanamycin–Pd complexes compared with free drugs and controls against M. tuberculosis H37Ra. ***, **, * statistically different at 95% significance level (n = 3) (adapted with permission from Ref. [72])*

In the case of metal complexes, albeit metal chelation can be advantageous in many ways, this strategy has to be carefully reasoned prior to being employed. Analysis of the cytotoxic effect of the capreomycin–, kanamycin– and ofloxacin–Pd complexes on human bronchial epithelial cells highlights the fact that metal complexation can significantly increase toxicity even in comparison with the metal alone (Figure 12.7). Nevertheless, the

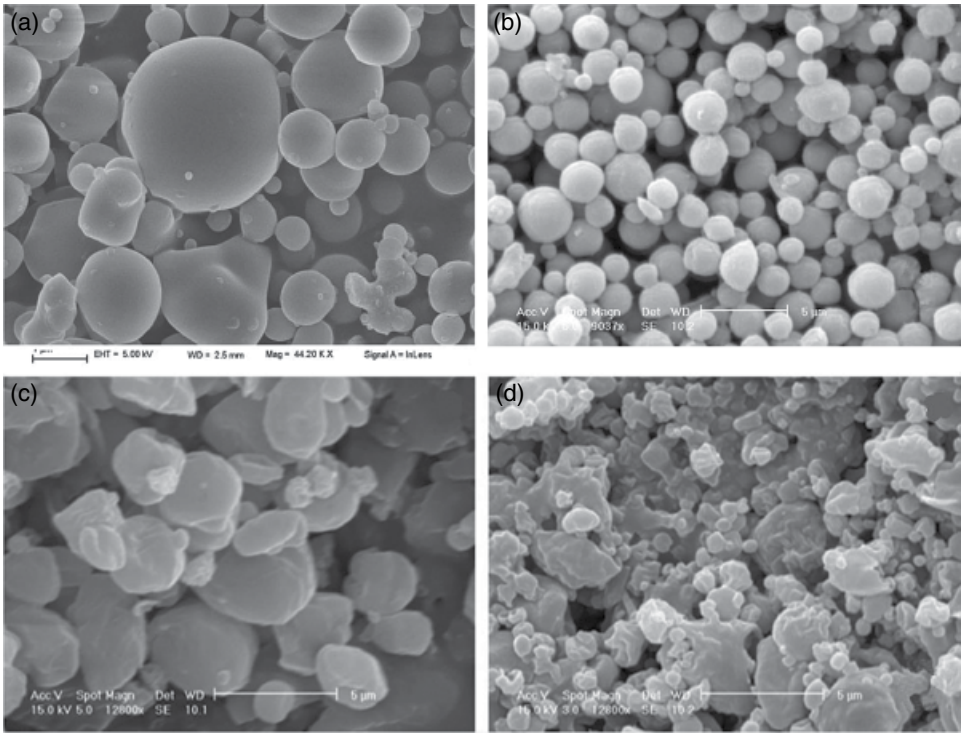


Figure 12.5 *Morphology of spray-dried polymeric microparticles loaded with (a) capreomycin–Pd, (b) ofloxacin–Pd complexes (adapted with permission from Ref. [74]). Spray-dried dry powders of capreomycin oleate ion pair obtained by (c) Buchi B290 mini spray-dryer and (d) B90 nano spray-dryer (adapted with permission from Ref. [75])*

Table 12.1 *Survival of chicken embryos treated with the different hydrophobic ion pairs on the chorioallantoic membrane (adapted with permission from Ref. [75])*

Time (hours)	Percentage survival (%)			
	CS ($n^a = 10$)	CO ($n^a = 10$)	SO ($n^a = 10$)	Negative control ($n^a = 4$)
0	100	100	100	100
24	70	100	10	100
48	50	70	0	75

CS, Capreomycin sulfate; CO, capreomycin oleate; SO, sodium oleate.
[a]n, Number of treated embryos.

drug concentrations at which significant toxicity was recorded are much higher than the estimated MIC (minimum inhibition concentration, 0.5–7 μg/mL). Furthermore, the equivalent Pd concentrations in the complex and alone are remarkably high (Pd = 220–370 μg/mL) if compared with other well-known toxic metals, such as Cd, that can be lethal to cells at concentrations <15 μg/mL [76]. This may allow theorizing that such

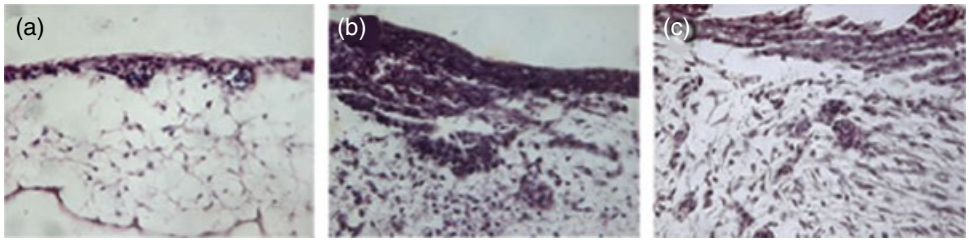


Figure 12.6 *Orthogonal histological sections of chicken embryo chorioallantoic membranes of (a) control group, (b) group treated with capreomycin sulfate and (c) group treated with capreomycin oleate ion pair at day 8. Magnification: 400× (adapted with permission from Ref. [75]). (See insert for color representation of the figure.)*

compounds could be useful when employed at low dosages and in extreme cases of multidrug resistance.

The intrinsic toxicity of heavy metals can be useful if seeking anticancer action but, if not accurately controlled, can result in serious side effects. The main issues in the use of metals account for cytotoxicity, genotoxicity, mutagenicity and bioaccumulation [77, 78]. The unclear fate of many such substances limits further their acceptability even when using metal microelements.

12.4 Remarks

The outlined scenario of the pharmaceutical application of the ion pair strategy enables several considerations to be undertaken. Albeit drug modification approaches have been employed for a century in different fields, only recently a deeper understanding of the therapeutic implications of ion pairing has suggested novel potentials for the improvement of current treatments. In particular, infectious disease therapies may benefit from new insights and progress and, in particular, the pulmonary treatment of such diseases seems to be the most logical step forward. Several reasons support this hypothesis, from the wide application of metal chelation and ion pairing to antibiotic drugs to the possibility to easily formulate inhalable dry-powders of such hydrophobic compounds. In this regard, TB infection seems to be the perfect target of such therapeutic solutions for at least two main reasons: the described ATD combination therapy flaws and the need for a more sustainable treatment as well as patient adherence.

To do so, efforts should be renewed towards the establishment of an effective inhalation therapy rather than the sole perpetual search for more potent, but even more toxic, ATDs. Moreover, being that ion paired drugs are not chemically modified, such an approach can favorably speed up bench-to-market processes.

Within such a framework, however, the assessment of toxicological profiles of such compounds is of paramount importance. Despite the large body of toxicological data on metals, this aspect is often neglected for ion pairs and complexes. Inconsistent data have been generated, especially when concerning inhalation, where, so far, little work has been done to expedite acceptance of novel excipients and materials for pulmonary drug delivery.

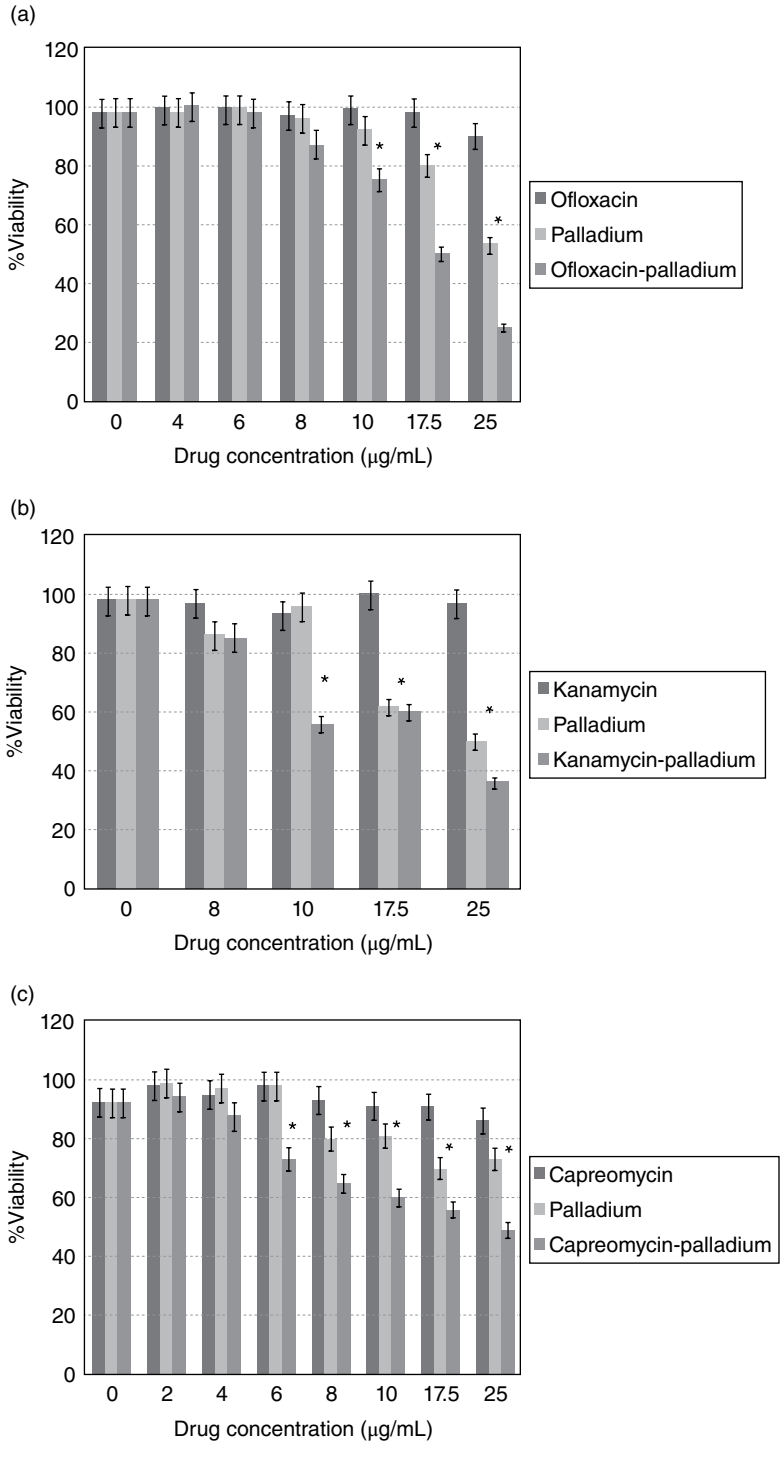


Figure 12.7 *Cytotoxicity on human bronchial epithelial cells (BEAS) at 24 hours after treatment with (a) ofloxacin–Pd, (b) kanamycin–Pd and (c) capreomycin–Pd complexes as compared with controls. *Statistically different at 95% significance level (n = 3). (S. Giovagnoli et al., unpublished data)*

References

[1] Marcus, Y. and Hefter, G. (2006) Ion pairing. *Chem. Rev.*, **106**, 4585–4621.
[2] Debye, P. and Huckel, E. (1923) The theory of electrolytes. I. Lowering of freezing point and related phenomena. *Phys. Z.*, **24**, 185–206.
[3] Onsager, L. (1927) The theory of electrolytes. II, *Phys. Z.*, **28**, 277–298.
[4] Bronsted, J.N. (1920) Studies on solubility. I. The solubility of salts in salt solutions. *J. Am. Chem. Soc.*, **42**, 761–786.
[5] Bjerrum, N. (1926) Ionic association. I. Influence of ionic association on the activity of ions at moderate degrees of association. *Kgl. Danske Videnskab. Selskab. Math.-fys. Medd.*, **7**(9), 1–48.
[6] Fuoss, R.M. (1934) Distribution of ions in electrolytic solution. *Trans. Faraday Soc.*, **30**, 967–980.
[7] Fuoss, R.M. (1958) Ionic association. III. The equilibrium between ion pairs and free ions. *J. Am. Chem. Soc.*, **80**, 5059–5061.
[8] Marcus, R.A. (1998) Ion pairing and electron transfer. *J. Phys. Chem. B*, **102**, 10071–10077.
[9] Chialvo A.A. and Simonson, J.M. (2007) Ion pairing and counterion condensation in aqueous electrolyte and polyelectrolyte solutions: Insights from molecular simulation. *J. Mol. Liq.*, **134**, 15–22.
[10] Manning G.S. (2011) Counterion condensation theory of attraction between like charges in the absence of multivalent counterions. *Eur. Phys. J. E*, **34**, 132–150.
[11] Powers, M.E., Matsuura, J., Brassell, J. *et al*. (1993) Enhanced solubility of proteins and peptides in nonpolar solvents through hydrophobic ion pairing. *Biopolymers*, **33**, 927–932.
[12] Adjei, A., Rao, S., Garren, J. *et al.* (1993) Effect of ion-pairing on l-octanol–water partitioning of peptide drugs. I: The nonapeptide leuprolide acetate. *Int. J. Pharm.*, **90**, 141–149.
[13] Koufopoulou, S.-A., Pistos, C., Giaginis, C. and Tsantili-Kakoulidou, A. (2006) Application of the ion pair concept to the n-octanol–water partitioning of cefepime and cefpirome. *Int. J. Pharm.*, **316**, 52–57.
[14] Benaouda, F., Brown, M.B., Shah, B. *et al.* (2012) The influence of self-assembling supramolecular structures on the passive membrane transport of ion-paired molecules. *Int. J. Pharm*, **439**, 334–341.
[15] Lengsfeld, C.S., Pitera, D., Manning, M. and Randolph, T.W. (2002) Dissolution and partitioning behavior of hydrophobic ion-paired compounds. *Pharm. Res.*, **19**(10), 1572–1576.
[16] Meyer J.D. and Manning, M.C. (1998) Hydrophobic ion pairing: altering the solubility properties of biomolecules. *Pharm. Res.*, **15**(2), 188–193.
[17] Kendrick, B.S., Meyer, J.D., Matsuura, J.E. *et al.* (1997) Hydrophobic ion pairing as a method for enhancing structure and activity of lyophilized subtilisin BPN' suspended in isooctane. *Arch. Biochem. Biophys.*, **347**(1), 113–118.
[18] Arct, J., Chelkowska, M., Kasiura, K. and Pietrzykowski, P. (2002) The fatty acids as penetration enhancers of amino acids by ion pairing. *Int. J. Cosmetic Sci.*, **24**, 313–322.
[19] Quintanar-Guerrero, D., Allémann, E., Fessi, H. and Doelker, E. (1997) Applications of the ion-pair concept to hydrophilic substances with special emphasis on peptides. *Pharm. Res.*, **14**(2), 119–127.
[20] Esbjörner, E.K., Lincoln, P. and Nordén, B. (2007) Counterion-mediated membrane penetration: Cationic cell-penetrating peptides overcome Born energy barrier by ion-pairing with phospholipids. *Biochim. Biophys. Acta*, **1768**, 1550–1558.
[21] Claffey, D.J., Meyer, J.D., Beauvais, R. *et al.* (2000) Long chain arginine esters: a new class of cationic detergents for preparation of hydrophobic ion-paired complexes. *Biochem. Cell Biol.*, **78**(1), 59–65.
[22] Abraham, J.J. (1948) Some account of the history of the treatment of syphilis. *Br. J. Vener. Dis.*, **24**, 153–161.
[23] Wheate, N.J., Walker, S., Craig, G.E. and Oun, R. (2010) The status of platinum anticancer drugs in the clinic and in clinical trials. *Dalton Trans.*, **39**(35), 8113–8127.
[24] Edwards-Jones, V. (2009) The benefits of silver in hygiene, personal care and healthcare. *Lett. Appl. Microbiol.*, **49**(2), 147–152.

[25] Milacic, V., Fregona, D. and Dou, Q.P. (2008) Gold complexes as prospective metal-based anticancer drugs. *Histol. Histopathol.*, **23**, 101–108.
[26] Gasser, G., Ott, I. and Metzler-Nolte, N. (2011) Organometallic anticancer compounds. *J. Med. Chem.*, **54**, 3–25.
[27] Eze, F.I., Ajali, U. and Ukoha, P.O. (2014) Synthesis, physicochemical properties, and antimicrobial studies of iron(III) complexes of ciprofloxacin, cloxacillin, and amoxicillin. *Int. J. Med. Chem.*, **2014**, Article ID 735602.
[28] Waziri, I., Ndahi, N.P. and Paul, B.B. (2013) Synthesis, physicochemical and antimicrobial studies of Co(II), Zn(II) and Fe(III) mixed antibiotics metal complexes. *J. Chem. Pharm. Res.*, **5**(9), 84–89.
[29] Muthukumar, C., Sabastiyan, A., Ramesh, M. *et al.* (2012) Synthesis, physico-chemical characterization and antimicrobial studies on 7-diethylaminosalicyl-8-hydroxyquinoline and its metal complexes. *Int. J. ChemTech. Res.*, **4**(4), 1322–1328
[30] Hossain, Md.A., Islam, M.S., Alam, Md.A. and Sultan, T. (2013) Synthesis, physiochemical studies and antimicrobial screening of metal complexes of Fe(III) & Au(III) with amino acids. *Int. J. Sci. Technol. Res.*, **2**(7), 210–217.
[31] Auda, S.H., Mrestani, Y., Nies, D.H. *et al.* (2009) Preparation, physicochemical characterization and biological evaluation of cefodizime metal ion complexes. *J. Pharm. Pharmacol.*, **61**, 1–6.
[32] Thakur, D., Mishra, J., Pitre, K.S. and Joshi, S. (2013) Fe(III)-Chloroquine Complex: A New Potent Compound in Wellness Industries of High Antimicrobial and Antimalarial Activities. *Int. J. Biotechnol. Well. Ind.*, **2**, 140–144.
[33] Verma, S, Shrivastva, S. and Shrivastva, R. (2013) Synthesis, Characterization, and Physicochemical Studies of Mixed Ligand Complexes of Inner Transition Metals with Lansoprazole and Cytosine. *J. Chem.*, **2013**, Article ID 309179.
[34] Buchner, R., Samani, F., May, P.M. *et al.* (2003) Hydration and ion pairing in aqueous sodium oxalate solutions. *Chem. Phys. Chem.*, **4**(4), 373–378.
[35] ElShaer, A., Hanson, P. and Mohammed, A.R. (2014) A novel concentration dependent amino acid ion pair strategy to mediate drug permeation using indomethacin as a model insoluble drug. *Eur. J. Pharm. Sci.*, **62**, 124–131.
[36] Samiei, N., Mangas-Sanjuan, V., González-Álvarez, I. *et al.* (2013) Ion-pair strategy for enabling amifostine oral absorption: Rat in situ and in vivo experiments. *Eur. J. Pharm. Sci.*, **49**, 499–504.
[37] Samiei, N., Shafaati, A., Zarghi, A. *et al.* (2014) Enhancement and in vitro evaluation of amifostine permeation through artificial membrane (PAMPA) via ion pairing approach and mechanistic selection of its optimal counter ion. *Eur. J. Pharm. Sci.*, **51**, 218–223.
[38] Miller, J.M., Dahan, A., Gupta, D. *et al.* (2010) Enabling the intestinal absorption of highly polar antiviral agents: ion-pair facilitated membrane permeation of zanamivir heptyl ester and guanidino oseltamivir. *Mol. Pharm.*, **7**, 1223–1234.
[39] Liu, K.-C., Lee, P.-S., Wang, S.-Y. *et al.* (2011) Intramolecular ion-pair prodrugs of zanamivir and guanidino-oseltamivir. *Bioorg. Med. Chem.*, **19**, 4796–4802.
[40] You, S.K., Kwon, H.H., Lee, J.M. *et al.* (2009) Studies on the formation of hydrophobic ion-pairing complex of alendronate. *Arch. Pharm. Res.*, **32**, 1055–1060.
[41] Sun, S., Liang, N., Kawashima, Y. *et al.* (2011) Hydrophobic ion pairing of an insulin-sodium deoxycholate complex for oral delivery of insulin. *Int. J. Nanomed.*, **6**, 3049–3056.
[42] Vadlapatla, R., Fifer, E.K., Kim, C.J. and Alexander, K.S. (2009) Drug-organic electrolyte complexes as controlled release systems. *Drug Dev. Ind. Pharm.*, **35**, 1–11.
[43] Jacob, G. (2013) Synthesis, Physico-chemical and antidiabetic studies of zinc complex of glimepiride, an oral hypoglycemic agent. *Orient. J. Chem.*, **29**(4), 1351–1358.
[44] Valenta, C., Siman, U., Kratzel, M. and Hadgraft, J. (2000) The dermal delivery of lignocaine: influence of ion pairing. *Int. J. Pharm.*, **197**, 77–85.
[45] Xi, H., Cun, D., Wang, Z. *et al*. (2012) Effect of the stability of hydrogen-bonded ion pairs with organic amines on transdermal penetration of teriflunomide. *Int. J. Pharm.*, **436**, 857–861.
[46] Xi, H., Wang, Z., Chen, Y. *et al.* (2012) The relationship between hydrogen-bonded ion-pair stability and transdermal penetration of lornoxicam with organic amines. *Eur. J. Pharm. Sci.*, **47**, 325–330.

[47] Kamal, M.A.H.M., Iimura, N., Nabekura, T. and Kitagawa, S. (2006) Enhanced skin permeation of salicylate by ion-pair formation in non-aqueous vehicle and further enhancement by ethanol and l-menthol. *Chem. Pharm. Bull.*, **54**, 481–484.
[48] Megwa, S.A., Cross, S.E., Whitehouse, M.W. *et al*. (2000) Effect of ion pairing with alkylamines on the in-vitro dermal penetration and local tissue disposition of salicylates. *J. Pharm. Pharmacol.*, **52**, 929–940.
[49] Trotta, M., Ugazio, E., Peira, E. and Pulitano, C. (2003) Influence of ion pairing on topical delivery of retinoic acid from microemulsions. *J. Control. Release*, **86**, 315–321.
[50] Ivaturi, V.D. and Kim, S.K. (2009) Enhanced permeation of methotrexate in vitro by ion pair formation with L-arginine. *J. Pharm. Sci.*, **98**, 3633–3639.
[51] Yeo, Y. and Park, K. (2004) Control of encapsulation efficiency and initial burst in polymeric microparticle systems. *Arch. Pharm. Res.*, **27**, 1–12.
[52] Allison, S.D. (2008) Analysis of initial burst in PLGA microparticles. *Expert Opin. Drug Deliv.*, **5**, 615–628.
[53] Teekamp, N., Duque, L.F., Frijlink, H.W. et al. (2015) Production methods and stabilization strategies for polymer-based nanoparticles and microparticles for parenteral delivery of peptides and proteins. *Expert Opin. Drug Deliv.*, **19**, 1–21.
[54] Estey, T., Kang, J., Schwendeman, S.P. and Carpenter, J.F. (2006) BSA degradation under acidic conditions: a model for protein instability during release from PLGA delivery systems. *J. Pharm. Sci.*, **95**, 1626–1639.
[55] L. Yang, L., F. Cui, F., K. Shi, K. *et al.* (2009) Design of high payload PLGA nanoparticles containing melittin/sodium dodecyl sulfate complex by the hydrophobic ion-pairing technique. *Drug Dev. Ind. Pharm.*, **35**, 959–968.
[56] Rastogi, R., Anand, S. and Koul, V. (2009) Evaluation of pharmacological efficacy of 'insulin–surfoplex' encapsulated polymer vesicles. *Int. J. Pharm.*, **373**, 107–115.
[57] Choi, S.H. and Park, T.G. (2000) Hydrophobic ion pair formation between leuprolide and sodium oleate for sustained release from biodegradable polymeric microspheres. *Int. J. Pharm.*, **203**, 193–202.
[58] R. Gaudana, R., M. Gokulgandhi, M., V. Khurana, V. *et al*. (2013) Design and evaluation of a novel nanoparticulate-based formulation encapsulating a HIP complex of lysozyme. *Pharm. Dev. Technol.*, **18**, 752–759.
[59] Dai, W.-G. and Dong, L.C. (2007) Characterization of physiochemical and biological properties of an insulin/lauryl sulfate complex formed by hydrophobic ion pairing. *Int. J. Pharm.*, **336**, 58–66.
[60] Gaudana, R., Khurana, V., Parenky, A. and Mitra, A.K. (2011) Encapsulation of protein-polysaccharide hip complex in polymeric nanoparticles. *J. Drug Deliv.*, **2011**, Article ID 458128.
[61] Alcock, R., Blair, J.A., O'Mahony, D.J. *et al*. (2002) Modifying the release of leuprolide from spray dried OED microparticles. *J. Control. Release*, **82**, 429–440.
[62] Patel, A., Gaudana, R. and Mitra, A.K. (2014) A novel approach for antibody nanocarriers development through hydrophobic ion-pairing complexation. *J. Microencapsul.*, **31**, 542–550.
[63] Imbuluzqueta, E., Elizondo, E., Gamazo, C. *et al*. (2011) Novel bioactive hydrophobic gentamicin carriers for the treatment of intracellular bacterial infections. *Acta Biomater.*, **7**, 1599–1608.
[64] Yang, L., Luo, J., Shi, S. *et al*. (2013) Development of a pulmonary peptide delivery system using porous nanoparticle-aggregate particles for systemic application. *Int. J. Pharm.*, **451**, 104–111.
[65] Blasi, P., Schoubben, A., Giovagnoli, S. *et al*. (2009) Fighting tuberculosis: old drugs, new formulations. *Expert Opin. Drug Deliv.*, **6**(9), 977–993.
[66] Gupta, A., Pandya, S.M., Mohammad, I. *et al.* (2013) Particulate pulmonary delivery systems containing anti-tuberculosis agents. *Crit. Rev. Ther. Drug Carrier Syst.*, **30**(4), 277–291.
[67] Misra, A., Hickey, A.J., Rossi, C. *et al*. (2011) Inhaled drug therapy for treatment of tuberculosis. *Tuberculosis*, **91**, 71–81.
[68] Hoppentocht, M., Hagedoorn, P., Frijlink, H.W. and de Boer, A.H. (2014) Developments and strategies for inhaled antibiotic drugs in tuberculosis therapy: A critical evaluation. *Eur. J. Pharm. Biopharm.*, **86**, 23–30.

[69] Zhou, H., Lengsfeld, C., Claffey, D.J. *et al*. (2002) Hydrophobic ion pairing of isoniazid using a prodrug approach. *J. Pharm. Sci.*, **91**, 1502–1511.
[70] Zhou, H., Zhang, Y., Biggs, D.L. *et al.* (2005) Microparticle-based lung delivery of INH decreases INH metabolism and targets alveolar macrophages. *J. Control. Release*, **107**, 288–299.
[71] Vieira, L.M.M., de Almeida, M.V., Lourenco, M.C.S. *et al.* (2009) Synthesis and antitubercular activity of palladium and platinum complexes with fluoroquinolones. *Eur. J. Med. Chem.*, **44**, 4107–4111.
[72] Giovagnoli, S., Marenzoni, M.L., Nocchetti, M. *et al*. (2014) Synthesis, characterization and in vitro extracellular and intracellular activity against Mycobacterium tuberculosis infection of new second-line antitubercular drug-palladium complexes. *J. Pharm. Pharmacol.*, **66**, 106–121.
[73] Palazzo, F., Giovagnoli, S., Schoubben, A. *et al*. (2013) Development of a spray-drying method for the formulation of respirable microparticles containing ofloxacin–palladium complex. *Int. J. Pharm.*, **440**, 273–282.
[74] Giovagnoli, S., Palazzo, F., Di Michele, A. *et al*. (2014) The influence of feedstock and process variables on the encapsulation of drug suspensions by spray-drying in fast drying regime: the case of novel antitubercular drug–palladium complex containing polymeric microparticles. *J. Pharm. Sci.*, **103**, 1255–1268.
[75] Schoubben, A., Blasi, P., Marenzoni, M.L. et al. (2013) Capreomycin supergenerics for pulmonary tuberculosis treatment: Preparation, in vitro, and in vivo characterization. *Eur. J. Pharm. Biopharm.*, **83**, 388–395.
[76] Luca, G., Lilli, C., Bellucci, C. *et al*. (2013) Toxicity of cadmium on sertoli cells functional competence: an in vitro study. *J. Biol. Regul. Homeostatic Agents*, **27**(3), 805–816.
[77] European Medicines Agency for the Evaluation of Medicines for Human Use (2007) Guideline on the specification limits for residues of metal catalysts. Doc. Ref. CPMP/SWP/QWP/4446/00, London, January 2007. (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003587.pdf) (accessed 8th April 2016).
[78] Martin, S. and Griswold, W. (2009) Human Health Effects of Heavy Metals. *Environmental Science and Technology Briefs for Citizens*, **15**, 1–6. (http://www.engg.ksu.edu/chsr/files/chsr/outreach-resources/15HumanHealthEffectsofHeavyMetals.pdf) (accessed 8th April 2016).

13

Understanding the Respiratory Delivery of High Dose Anti-Tubercular Drugs

Shyamal C. Das[1] *and Peter J. Stewart*[2]

[1] *School of Pharmacy, University of Otago, Adams Building, Dunedin, New Zealand*
[2] *Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia*

13.1 Introduction

The purpose of this chapter is to consider the respiratory delivery of drugs used in the treatment of tuberculosis. The chapter will provide a rationale for respiratory delivery of anti-tubercular drugs to the lungs, including the targeting of alveolar macrophages and granulomas. The doses of anti-tubercular drugs are high and most formulations will be carrier free containing drug and limited amounts of excipient. The aerosolization of high dose drugs existing in a cohesive matrix will be related to de-agglomeration processes. A theoretical basis is provided to identify the parameters that will influence the de-agglomeration and aerosolization of these drugs from cohesive matrices. The chapter will outline the studies that have occurred in this area using particle engineering approaches.

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

13.2 Tuberculosis

Tuberculosis (TB) is an infectious disease caused by the bacterium *Mycobacterium tuberculosis* (MTb). Although the disease is curable with proper treatment and is preventable with vaccination, more than a hundred million people have died of tuberculosis in the last hundred years. Treatment and vaccinations are available; however, still around one third of the world's current population (two billion) is infected with MTb [1, 2]. In 2013, nine million new cases were reported and one and a half million people died [3].

There are two types of TB based on the anatomical location: pulmonary TB (PTB) and extrapulmonary TB (EPTB). When MTb is localized in the lung, TB is called PTB, and when MTb is spread over other organs beyond the lung such as lymph nodes, abdomen, pleura, joints, bones, skins, genitourinary tract and meninges, TB is called EPTB [4]. PTB accounts for 75–80% of all TB.

TB can be identified by certain signs and symptoms which vary between PTB and EPTB. In general, a TB patient will have fever, loss of appetite, unintentional weight loss, chills and night sweats. In addition, PTB patients will have coughing for a minimum of three weeks with blood and sputum. They will experience pain in the chest or pain during breathing and coughing. EPTB patients will have different symptoms based on the organs that are affected. For example, back pain is common if the spine is affected while blood in the urine is common when kidneys are affected.

A person can be infected by MTb by inhaling droplets from an infected person. However, not all people who inhale MTb show the symptoms of TB immediately. Microorganisms are phagocytized by alveolar macrophages (AM) where two processes compete and determine the fate of the phagosomal MTb (Figure 13.1). In an immune-competent person, the

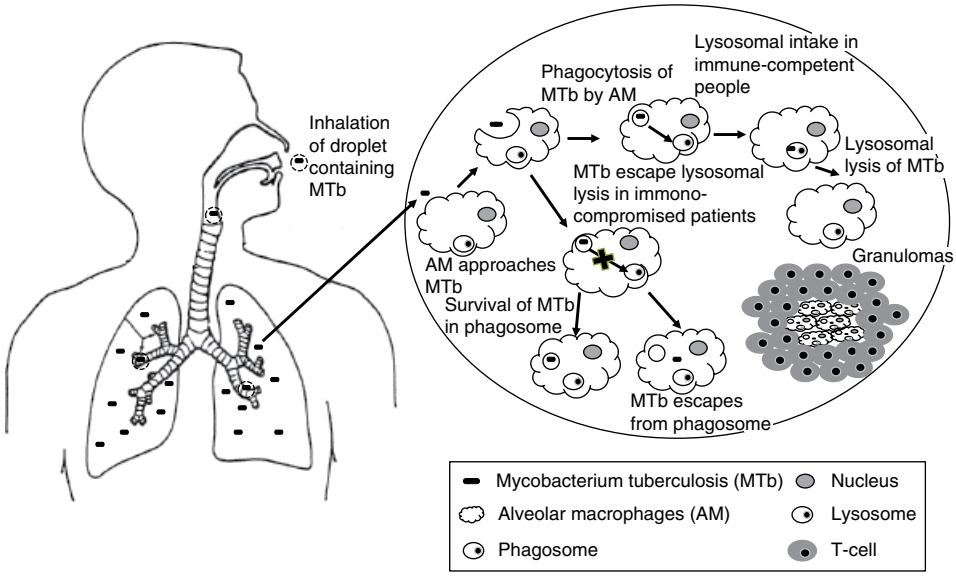


Figure 13.1 *Infection of Mycobacterium tuberculosis and entry into alveolar macrophages (AM) by phagocytosis and the formation of granulomas [5]*

MTb will be killed by phagosome–lysosome fusion and lysosomal lysis, and multiplication of MTb will be inhibited. On the other hand, if the infected person is immune-compromised, the MTb will resist phagosome–lysosome fusion and lysosomal lysis. The MTb will create an environment of 'alternative activation' so that they can multiply [1]. A granuloma is formed with accumulation of myeloid cells in its centre and lymphatic cells on the outer layer. The central part is necrotic in nature, hypoxic and contains very low levels of nitric oxide. This environment is excellent for MTb survival. In this condition, MTb can survive for years. This condition of TB is called latent TB [6]. Around 80–90% of granulomas are localized in the lung. They are poorly vascularized and difficult to access by anti-TB drugs. This, together with spontaneous mutation of MTb, helps to develop drug resistance [7]. In suitable conditions, the granulomas can rupture and MTb starts multiplying, causing active TB [8]. Mortality due to TB has recently worsened because of the (a) comorbidity from Acquired Immuno-Deficiency Syndrome (AIDS) and (b) emergence of multi-drug-resistant TB (MDR-TB) [9], extensively drug-resistant TB (XDR-TB) [10] and totally drug-resistant TB (TDR-TB). MDR-TB is defined if the bacterium is resistant to isoniazid and rifampicin, while XDR-TB is defined if the bacterium is resistant to isoniazid, rifampicin, fluoroquinolones and at least one injectable second-line drug such as amikacin, capreomycin or kanamycin [4]. TDR-TB means complete resistance to all available drugs.

13.3 Drugs Used to Treat Tuberculosis, Doses, Challenges and Requirements for Therapy in Lungs

13.3.1 Current TB Treatment Regimen

The treatment of TB requires a number of drugs to be taken several times a day for months to years. These drugs are classified as first-line and second-line drugs. The treatment starts with first-line drugs that include isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin. All the first-line drugs are currently taken orally except streptomycin, which is administered by injection. Second-line drugs are included in the regimen when a patient develops resistance to certain drugs. Different types of second-line drugs include injectable agents such as kanamycin, amikacin and capreomycin; fluoroquinolones such as levofloxacin, ofloxacin and moxifloxacin; other oral bacteriostatic agents such as *p*-aminosalicylic acid, cycloserine, terizidone, ethionamide and prothionamide; and agents with unclear role in drug-resistant TB such as clofazimine, linezolid, amoxicillin/clavulanate, thioacetazone, imipenem/cilastatin and clarithromycin. In general, second-line drugs are more toxic in nature [11].

The selection of drugs and duration of treatment depend on the type of MTb. The treatment time is primarily six months for drug-susceptible TB which includes the intensive phase of treatment of two months followed by a continuous phase of treatment of four months [11]. During the intensive phase, a patient needs to take isoniazid, rifampicin and ethambutol daily while the same patient needs to continue with two drugs, isoniazid and rifampicin, three times a week in their continuous phase.

For MDR-TB, the World Health Organization (WHO) recommends a combination of at least four drugs for the treatment to be effective [11]. It is recommended to choose one drug from a group of oral first-line drugs such as pyrazinamide, ethambutol and rifabutin and the

other drugs are from second-line drugs: one drug should be from injectable agents, one drug from the fluoroquinolones, and one or more from oral bacteriostatic agents. The total number of drugs to be taken can be up to seven. The intensive phase of MDR-TB treatment is six months, followed by a continuation phase of four months if the patient shows as being smear or culture negative. The patient should be treated for 18–24 months after culture conversion.

13.3.2 Challenges of Conventional Oral and Parenteral Therapy

The challenges of current TB treatment using oral and parenteral agents are:

a. Long duration of treatment: six months for drug-susceptible TB to 24 months for MDR-TB.
b. Large number of tablets and injections: An MDR patient needs to take up to 22 tablets and 4 injections each day, which is approximately 3000 injections and 22,000 tablets in two years.
c. Frequency of dosing: Most of the oral tablets need to be taken 3 to 4 times a day during their intensive phases.
d. Dose of drugs is high: The oral or even parenteral doses of the anti-TB drugs are high. For example, pyrazinamide oral tablet contains 400 mg of active ingredient per tablet, while each vial of streptomycin or kanamycin contains 1 g of powder as sulfate. Altogether a patient needs to take 2–3 g of drugs daily during the intensive phases of treatment of drug-susceptible TB.
e. Drugs possess side effects: Most of the anti-TB drugs have toxic side effects. Side effects can range from mild nausea and vomiting by isoniazid to ototoxicity and nephrotoxicity of aminoglycosides kanamycin, amikacin, capreomycin and streptomycin. A list of side effects can be found in Das *et al*. [5].
f. Suboptimal level of drug in the lung: Since the drug is distributed throughout the body, a suboptimal amount of drug reaches the lung because of poor vascularization of granulomas and limited penetration of drugs to granulomas where the mycobacteria reside.
g. Poor adherence: Owing to the long duration of treatment, high frequency of dosing, severe side effects, high cost of medication and invasive delivery like injections, there is poor adherence to TB treatment. Suboptimal levels of drugs and poor adherence, in addition to spontaneous mutations of Mycobacteria, result in drug resistance [12].

13.3.3 Rationale for Respiratory Delivery

Since around 80% of TB is pulmonary TB, the direct delivery of drugs to the lung can be an efficient method of treatment. Local delivery of drugs to the lung has multiple advantages. First, respiratory delivery can result in the achievement of high concentrations of drugs in the lung achieved by direct delivery of drugs to the lung. Secondly, microparticles of drugs delivered to the lung are engulfed by alveolar macrophages. Thus, drugs can access alveolar macrophages where MTb resides in the lung. The engulfment of drugs by alveolar macrophages may rescue the alveolar macrophages from their alternative activation state to classical activation state so that the drugs can kill the MTb.

While pulmonary delivery of drugs is useful in treating PTB, it is also useful for treating EPTB. The drugs, absorbed into the systemic circulation through the lung epithelium, can

distribute throughout the body to target EPTB. The pulmonary route can also be used together with oral and parenteral routes to fortify the success of treatment.

13.4 Approaches for Respiratory Delivery of Drugs

Drugs can be delivered to the lung by three approaches, including metered-dose inhalers (MDIs), nebulizers and dry-powder inhalers (DPIs). Using MDIs, drugs are aerosolized from their solutions or suspensions in liquid propellants. The design of MDIs restricts the approach to delivering low doses of drugs for treating diseases such as asthma and chronic obstructive pulmonary disease (COPD); it is not capable of delivering a high drug dose and MDIs have not been used for the treatment of TB [2]. Drugs can be aerosolized as fine droplets from their solutions or suspensions using nebulizers [13, 14]. Nebulizers have been used for delivering drugs to young children or the elderly. Since a power source is necessary for operating traditional nebulizers, patients had to visit hospital two or three times a day according to their particular dosing regimen to inhale the relevant dose of the drug. The power source is noisy and the cleaning of the system after each delivery is time consuming. More recently, with the introduction of battery-operated handheld nebulizers, much of these drawbacks such as requirement of a power source and portability have been relieved; however, it is still a challenge to formulate a solution or suspension containing two or more drugs of different solubilities in the required proportions [2, 15, 16]. Moreover, drugs are in liquid form with potential stability problems and nebulization is still time consuming due to the delivery time and the requirements of regular cleansing with disinfectant. Thus nebulization is not the preferred method for high dose drug delivery [2, 17, 18]. In contrast, DPIs contain drugs in powder form which are, in general, more stable than the liquid forms that occur in MDIs and nebulizers. DPIs are more user friendly since they are portable and the formulation of poorly water-soluble drugs can be prepared easily. With proper design, DPIs can be used to deliver high doses of drugs. Although nebulizers are also researched, DPIs have been the major focus in recent times for delivering anti-TB drug powders.

The DPI system is a combination of a powder mixture containing drugs and the device. With proper design of the device containing appropriately formulated powder, dry-powder formulation for TB treatment is possible. In the following sections we will discuss the formulation of powders for inhalation in the treatment of TB.

13.5 Current DPI Formulations and Their Mechanisms of Aerosolization

DPIs have been used for the treatment of asthma and COPD for over four decades. There are two types of formulations of powders for inhalation: drug carrier-based formulation and drug in controlled agglomerates.

First, since the treatment of asthma and COPD requires very small doses of bronchodilators and corticosteroids, generally 6–400 μg, there is a challenge in metering this low amount of cohesive drug due to its poor flowability. In order to improve both flow property and increase the bulk, carrier-based formulations have been prepared where large-particulate-size carriers such as lactose are intentionally added to the drug (Figure 13.2a). In such formulations, the cohesive drug particles are attached to carriers and/or form agglomerates.

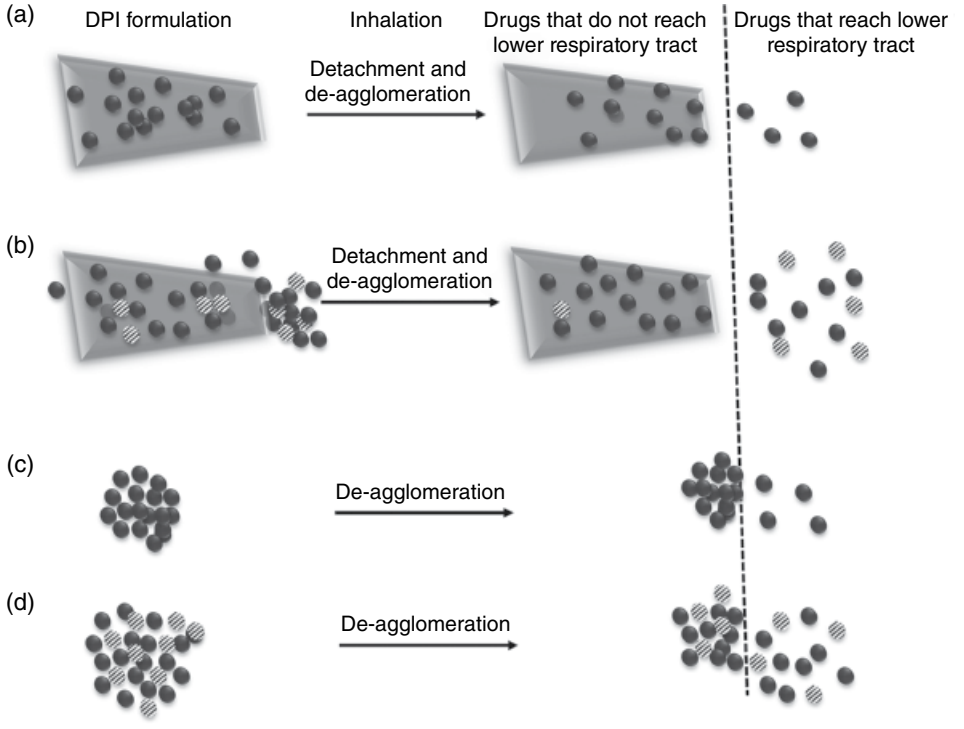


Figure 13.2 *Mechanism of aerosolization of drugs from carrier-based formulations without and with fine lactose (a and b, respectively) and drug agglomerates without and with fine lactose (c and d, respectively) (Drug = small black ball, fine lactose = small striped ball, large carrier = large grey quadrilateral)*

During inspiration, drug particles need to be detached from carriers and de-agglomerated to primary particles less than 5 μm in diameter to reach the lower respiratory tract. Therefore, two processes are required in the aerosolization of drugs in these formulations: detachment from carriers and drug de-agglomeration [18]. Improvement in the drug delivery efficiency to the lung has been achieved by adding ternary components such as fine lactose to the carrier-based formulations (Figure 13.2b). These ternary components were shown to increase dispersion (a) by passivating the active surface of large carriers so drugs are attached to lower energetic surfaces, thereby facilitating the detachment from carriers, and/or (b) by forming mixed agglomerates of drug and ternary carriers which will be de-agglomerated more easily. The de-agglomeration again depends on the detachment of drug/drug-agglomerates/drug–ternary carrier agglomerates and de-agglomeration of the agglomerates to primary drug particles [19]. The doses of current carrier-based formulations range from 6 μg for formoterol fumarate to 500 μg for fluticasone. The formulation contains very low concentrations of drugs, typically <4% [20]. A list of current drugs used for asthma and COPD with their dose is given in Table 13.1.

Secondly, powders for inhalation can be formulated as drug agglomerates which contain micronized (aerodynamic size of particles <5 μm) drugs only (Figure 13.2c) or drugs with

Table 13.1 *The doses of common drugs used for asthma and COPD*

Drug	Dose per puff (μg)	Number of puffs	Dose per time (μg)
Beta-2 agonists			
Salbutamol	100	2	200
Terbutaline	250	2	500
Salmeterol	50	1	50
Anticholinergic			
Tiotropium	18	1	18
Combination inhalers			
Budesonide and Eformoterol	200/6	2	400/12
Corticosteroid			
Budesonide	400	1	400
Fluticasone	250	2	500

fine lactose in agglomerates (Figure 13.2d) formed during a controlled agglomeration process. De-agglomeration is the principal mechanism of aerosolization of the drug-agglomerate formulations (Figure 13.2).

13.6 DPI Formulations for Tuberculosis and Requirements

The principal aim for tuberculosis treatment with DPIs is that adequate amounts of drug are available to kill MTb at their site of existence in the body. In active PTB patients, MTb resides in the lung, particularly in the alveolar macrophages. Drugs need to be available inside alveolar macrophages to kill MTb that reside in alveolar macrophages. MTb also reside in granulomas mainly during the dormant stage. Since the granulomas have a protective barrier and they are poorly vascularized, drugs need to enter the granuloma to kill MTb. For many first-line anti-TB drugs, the oral or parenteral doses are very high. For example, the oral dose of pyrazinamide per tablet is 400 mg and the parenteral injection of streptomycin contains 1 g of streptomycin sulfate. Although oral doses of anti-TB drugs are known, it is not clear how much drug will be needed for respiratory delivery to be effective against either drug-susceptible or drug-resistant TB. It is predicted that the dose of different drugs could be several milligrams to around a hundred milligrams [19]. Thus, the formulation of a powder for respiratory delivery will contain high doses of drugs and the formulation is likely to be drug alone or drug with small amounts of excipients (such as engineered particles) without carriers.

13.7 Issues to Consider in Respiratory Delivery of Powders for Tuberculosis

The following issues need to be considered in designing high dose drug powders for respiratory delivery in the treatment of TB.

a. Powder flow: The high dose powder mixture should have good flow to ensure easy processing of the powder, particularly filling of capsules or of the device, and efficient

delivery of the powder out of the capsule or device during inhalation. Ideally, the emitted dose should be close to 100% of that available. Highly flowable particles can be produced by particle engineering techniques including low-density particles by spray drying only drug [20], spray drying with materials such as leucine to assist flow [21] and mechanofusion using magnesium stearate [22].

b. Aerosolization of high dose powders: In order to deliver high doses of drugs, effective de-agglomeration of the cohesive powder matrix must occur. The factors that affect de-agglomeration, including the particle-size distribution of cohesive powders, the surface characteristics including its surface energy and the packing fraction of the cohesive matrix should be considered; these parameters are discussed in the section on de-agglomeration.
c. Alveolar macrophage uptake: Since MTb are initially phagocytized by AM and they reside inside AM by alternative activation, it is important that the drug particles are phagocytized by AM to enable effective drug concentrations at the target site in the AMs. It is claimed that AM can phagocytize 2–10 μm size particles with their preferred size being about 3 μm [19]. Since particles <5 μm in diameter are suitable for respiratory delivery and can be delivered to the alveolar region, particles between 2–5 μm are ideal for anti-TB drug powders. The shape and surface characteristics of particles are also important for optimum macrophage uptake [23].
d. Dissolution of drug particles: The mechanism of dissolution and the factors affecting dissolution rate are not well known for drug particles in the lungs. The alveolar region has a large surface area (>100 m^2) and contains a very limited amount of fluid typically calculated as ~15 ml (it may vary from 7 to 70 ml depending on the specific approaches to calculating this volume). This aqueous fluid contains high concentrations of lung surfactants that are stored as lamellar bodies after being secreted by type II alveolar cells. These lamellar bodies then unravel in the alveolar space, forming tubular myelins which are similar to multi-lamellar liposomes. In some studies, the existence of nanostructures such as inverse hexagonal phases has been assumed [24–26]. Although there was no direct evidence, the presence of other nanostructures such as inverse cubic phases has also been predicted [27]. The role of these nanostructures on the dissolution and delivery of anti-TB drugs is not clear.

   It also is unclear whether the dissolution of drugs in the lung is diffusion controlled or permeation controlled. Drug particles deposit in the aqueous fluid of the alveoli. Inhaled drug particles first contact a lung surfactant monolayer which is spread on an aqueous sub-phase overlying the respiratory epithelium. The drug particles need to cross the lung surfactant barrier and then dissolve in the very small amount of lung fluid in order for them to be absorbed. It is not clear how lung surfactants interact with high doses of anti-TB drugs.

   The local concentration of drug is dependent on the number of particles (single or multi) depositing in a particular region and the particle concentration will influence the rate and mechanism of dissolution. Few data are available on the effects of administering high doses of drug to the lung and on their dissolution characteristics in the lung. Since the dissolved drug is subject to various mechanisms of clearance in the alveolar region, the residence time of a drug in the alveolar region to achieve optimum anti-TB outcome is unknown.
e. The pharmacokinetics of drugs: Once the drugs reach the alveolar region, drug particles are subject to clearance by alveolar macrophage uptake and dissolution of the drug particles.

Undissolved particles of an appropriate size will be engulfed by macrophages. Drug will be dissolved and a portion of the dissolved drug will be absorbed by epithelial cells where they might interact with complimentary receptors or they may be metabolized. Another portion of the dissolved drug will diffuse through the epithelial cells to blood and will be distributed to the whole body. Some parts of the dissolved drug will be diffused back to alveolar macrophages. Since alveolar macrophages harbour MTb, the principal target of drug formulation design should be to maximize the uptake by these AM.

f. Physical stability of powders: Powder formulation should maintain its physical stability during its shelf life. It should have adequate stability against moisture since a hygroscopic powder may absorb moisture during storage and compromise the de-agglomeration capacity.
g. Processing and formulation: The drug powders can be prepared by micronization, milling, and crystallization or by spray drying. The formulations can also involve a mixing process. Whatever the processing involved, the ultimate powder formulation should have good flow properties.
h. Relationship between device and formulation: For high dose delivery, the nature of the device is important. Aerosolization capacity varies widely among different devices. The selection of device requires consideration of several factors. It is important to choose the right device that can deliver the selected formulation efficiently. Moreover, the cost of the device is an important issue since TB is predominant in low-economy countries of Africa and Asia.

13.8 Relationship between De-agglomeration and Tensile Strength

Formulations of particles or engineered particles which possess a suitable size for respiratory delivery can be considered as cohesive matrices. These matrices can be represented diagrammatically as shown by Figure 13.3.

The cohesive matrix will possess a distribution of particles. Particles produced by milling processes such as micronization will always possess a distribution of particles and, while the objective of engineered particle production will be to produce a monosize distribution, in reality, a particle-size distribution will most often result.

The particles in the matrix will interact due to the various interactive forces that exist between particles, including contact potential forces, Coulombic forces, intermolecular forces, capillary force and solid bridging. These interactions have been described in previous literature [28]; however, the contact potential and Coulombic forces constitute the electrostatic forces, whereas the intermolecular forces will be related to van der Waals' forces and hydrogen bonding. The capillary and solid bridging will depend on the extent of adhered moisture and are most likely to occur at high humidity. The total interactive force between particles will be related to the contribution of each of these forces at any time and will be dependent on the properties of the material, powder processing and handling and the environment. Since the magnitude of these forces at any one time depends on the surface energy, area of contact and distance between particles, the total interactive force will be distributed.

The packing fraction of powders is the concentration of particles per volume of powder. If the particles are packed close to each other, then the packing fraction is high, and vice

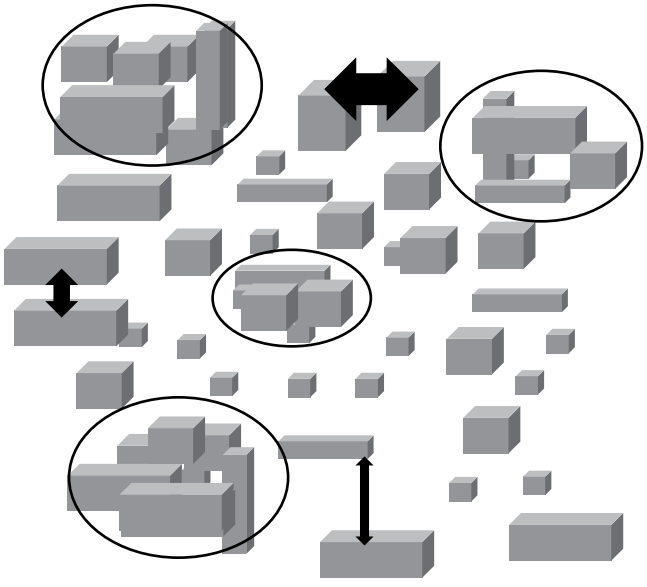


Figure 13.3 *Cohesive matrix of drug particles, prepared by milling or particle engineering approaches and showing a distribution of particle size, particle interaction and packing fraction*

versa. Also, the packing fraction is likely to vary in different domains of the powder matrix due to the processing history of powders such as the powder production and storage immediately after production.

Owing to the distributions of particle size, particle interactions and packing fraction, the powder matrix can be considered as a non-homogeneous cohesive matrix of different micro-domains. The distribution of the tensile strength of the micro-domains of the cohesive matrix provides the cohesive powder structure. The de-agglomeration is therefore related to the tensile strength distribution of the micro-domains [29].

Several theoretical relationships have been determined to correlate the tensile strength of the cohesive matrix to various properties of the matrix. These theoretical relationships are shown in Table 13.2.

The equation proposed by Kendall and Stainton [34] has been used to calculate the relationship between powder de-agglomeration and tensile strength of powders. According to this equation, the tensile strength is related to the particle diameter, the packing of the cohesive powder bed and cohesive forces. Das *et al*. have proposed a method of calculating the powder strength distribution using a Monte Carlo simulation [29].

Figure 13.4 shows the tensile strength distribution and the aerosolization performance of two model drugs (Drug 1 and Drug 2). An excellent relation was found between tensile strength distributions and aerosolization behaviour of two model drugs. The difference in the tensile strength distribution is clearly seen in Figure 13.4a. The aerosolization of the two drugs was consistent with the tensile strength of the two model drugs, with the drug with the higher tensile strength showing a reduced aerosolization behaviour particularly at higher air flow rates used in the laser diffraction particle sizing of the aerosol plume.

Table 13.2 *Theoretical relationships found in the literature to relate tensile strength to properties of cohesive matrices (d_p = particle diameter, φ = packing fraction, W = work of cohesion, σ = tensile strength, F = bonding force, k = coordination number, ε = void fraction, p = precompressive force, α = shape coefficient, ρ = density, L, L_1, m, f and b are constants)*

Equation	Equation no.	References
$\sigma = \left(\frac{1-\varepsilon}{\varepsilon}\right) * k_2 * \left(\frac{p}{d_p^2}\right)^m$	(13.1)	[30]
$log\,\sigma = L * \log\left(\alpha * \varphi * d_p^{-1}\right) + \log L_1$	(13.2)	[31,32]
$\sigma = \left(\frac{F*(1-\varepsilon)*k}{2\pi * d_{agglomerate}^2}\right) * \left(\frac{d_{agglomerate}^3}{d_p^3} - \frac{4f}{b^2}\right)$	(13.3)	[33]
$\sigma = \left(\frac{F*(1-\varepsilon)*k}{2d_{agglomerate}^2}\right) * \left(\frac{\rho_{tap}}{\rho_{particle} - \rho_{tap}}\right) * \left(\frac{d_{agglomerate}^3}{d_p^3} - \frac{4f}{b^2}\right)$	(13.4)	[33]
$\sigma = \frac{15.6\varphi^4 W}{d_p}$	(13.5)	[34]

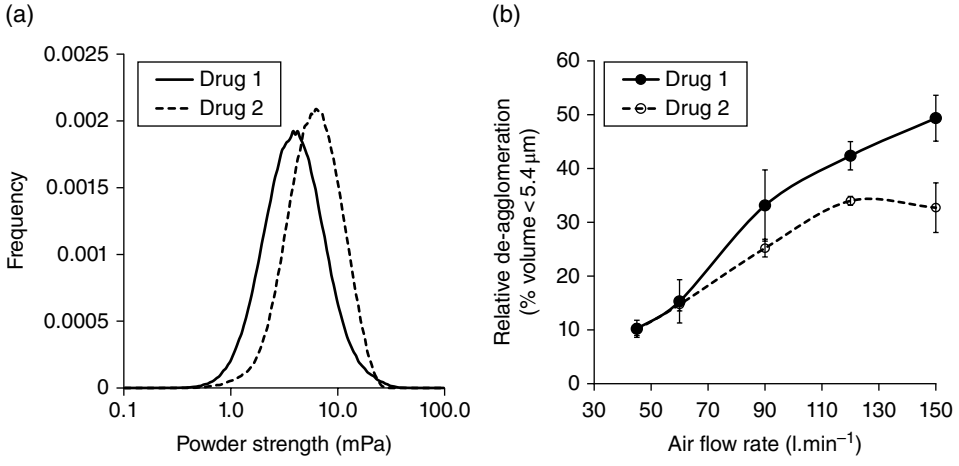


Figure 13.4 *Comparative tensile strength distributions (a) and aerosolization performance (b) of two model drugs*

13.9 Strategies to Improve De-agglomeration

In order to improve de-agglomeration, it is important to consider the factors affecting de-agglomeration and their manipulation to reduce tensile strength. According to the equation proposed by Kendall and Stainton [34], the tensile strength is affected by three factors: particle size, cohesive forces and packing fraction.

a. The tensile strength is inversely proportional to particle size. If we can increase particle size, keeping all others factors constant, the tensile strength will decrease, resulting in

improved de-agglomeration. However, since the particle size needs to be between 1 and 5 μm to enable efficient aerosolization and optimum phagocytosis by the AM, there is little scope of changing de-agglomeration by manipulating particle size. It is also difficult to produce size distributions of particles within that inhalable range that can offer a significant variation in aerosolization.

b. The tensile strength is directly proportional to work of cohesion. If we can decrease the cohesion between particles keeping all other factors constant, the tensile strength will decrease and consequently the de-agglomeration will increase. Various strategies have been used to change the surface characteristics, including coating the particle using conventional coating technologies, intensive dry coating techniques [22] and particle engineering approaches. The aim of these technologies is to reduce the cohesive interaction between particles in the particulate matrix, thus increasing aerosolization. Intensive dry coating or mechanofusion has been shown to be promising in this respect. Mechanofusion is a process of dry coating where a thin coat of guest particles is formed on host particles due to high shear mixing [22].

   Spray drying of drug particles with hydrophobic and low-cohesion material can produce powders which will have low cohesiveness, and high flowability. Using spray drying techniques, the drug salbutamol sulfate (SS) was coated with different concentrations of low-cohesion leucine, and the coating of SS with around 10% leucine has shown significant increase in both emitted dose and fine-particle fraction [35, 36]. In a recent study, Zhou *et al.* showed that co-spray drying of the antibiotic colistin with rifampicin increased the drug delivery efficiency significantly [37].

c. Packing fraction: The packing fraction is the most dominating factor that can affect tensile strength and, therefore, de-agglomeration as the tensile strength increases as the fourth power of packing fraction. In a study reported by Das *et al.* [38] the aerosolization behaviour of three lactoses was found to be different. The work of cohesion of the three powders was identical, particle sizes of cohesive fraction 5.4–14 μm were almost identical but they had significantly different packing fractions. This difference in packing fraction was due to differing proportions and shapes of intermediate cohesive fractions (5.4–14 μm). This packing-fraction difference contributed to the difference in tensile strengths and consequently their aerosolization [39].

13.10 DPI Formulations having High Aerosolization

High aerosolization efficiency of anti-TB dry-powder formulations was achieved by spray drying of drug alone. For example, fine-particle fractions (FPFs) of around 83 and 68% were achieved with crystalline and amorphous forms of rifapentine, respectively, produced by spray drying [40]. In another study by Young *et al*., an FPF value of around 67% was attained when ciprofloxacin was dispersed by Orbital®, a multi-dose inhaler tested by the group [41]. Son and McConville [42] developed an innovative spray drying method for producing respirable rifampicin. They spray dried both rifampicin and rifampicin dihydrate after recrystallization and reported 94% emitted dose (ED) and 50% FPF when rifampicin was dispersed using Handihaler. For rifampicin dihydrate, they reported 95 and 98% ED and 60 and 68% FPF determined using Handihaler and Aerolizer, respectively. High FPF values were also achieved with powders generated by spray drying and double emulsification. For example, ED and FPF were 57 and 55%, respectively, when isoniazid was spray dried alone [43].

Co-spray drying of two or more drugs often results in high FPF. For example, an ED of 96% and FPF of 92% were reported when colistin was co-spray dried with rifampicin [44]. Although the FPF was low (46%), around 89% ED was achieved for a spray dried triple combination of isoniazid, rifampicin and pyrazinamide [20].

The drug is often spray dried with a minimal amount of low-cohesive excipient such as leucine to improve aerosolization. For example, the FPF of a rifapentine-containing triple combination (rifapentine, moxifloxacin and pyrazinamide) increased from 56 to 64% when the combination was co-spray dried with 10% leucine [21]. The drug co-spray dried with dipalmitoylphosphatidylcholine (DPPC) has also shown an improvement in both ED and FPF, probably by reducing cohesiveness and powder density. For example, co-spray drying of moxifloxacin with DPPC, and ofloxacin with DPPC, resulted in an ED of 86 and 97% where the FPF was 67 and 68%, respectively [45].

Co-spray drying of drugs with mannitol has shown higher ED values although the FPF is not very high. For example, the ED was more than 90% for both tobramycin and azithromycin when each of them was co-spray dried with mannitol, but their FPF was less than 40% in each case [46]. This increase in ED was probably due to a change in shape to ball-like structures.

Drugs co-spray dried with biodegradable polymer have also shown improved efficiency. For example, rifampicin microparticles produced by spray drying and solvent evaporation with poly(lactic-co-glycolic acid) (PLGA) and leucine gave a FPF value of 67% [47], while rifampicin flash frozen with PLGA and mannitol has shown FPF values of 54% [48].

The results of studies using particle engineering approaches are summarized in Table 13.3.

13.11 Devices for High Dose Delivery

While high aerosolization was achieved by some techniques, overcoming dose limitations is an important consideration. The majority of current DPIs were developed for delivering low doses of drugs especially for treating asthma and COPD. High dose delivery is usually achieved using a single-dose reusable device rather than multi-dose inhalers since multi-dose inhaler devices would need to contain large amounts of drug.

Research is ongoing to develop devices that can deliver high doses of drugs to the lung. Currently, TOBI® Podhaler™, which is a passive DPI, is used to deliver 28 mg of tobramycin from a capsule containing 45 mg of powder. In this case, to deliver a dose of 112 mg of powder, a patient needs to inhale drugs from four capsules [51]. The Twincer® has been shown to de-agglomerate 25 mg of powder effectively while de Boer *et al*. suggest that this could be further optimized for 50 mg of powder [52,53]. A disposable device TwinCaps® DPI can be used to deliver a single dose of 40 mg of a long-acting neuraminidase inhibitor, laninamivir (Inavir®), for the treatment of influenza [54]. Multiple inhalation techniques can be used to deliver a high dose. For example, multiple inhalations are required to inhale 125 mg of colistimethate from the Colobreathe® Turbospin® device [55]. Using Orbital®, Young *et al.* showed that around 200 mg of powder can be delivered to patients in fewer than 10 inhalations [41].

Table 13.3 *Spray dried formulations of anti-TB drugs with high delivery efficiency*

Drugs	Formulations/Techniques	Aerosolization	References
Rifapentine	Spray drying	FPF 83% and 68% for crystalline and amorphous, respectively	[40]
Ciprofloxacin	Spray drying	FPF 67%	[41]
Rifampicin	Spray drying after recrystallization	ED 94% and FPF 50% using Handihaler	[42]
Rifampicin dihydrate	Spray drying after recrystallization	ED 95% and FPF 60% using Handihaler ED 98% and FPF 68% using Aerolizer	[42]
Isoniazid	Spray drying and double emulsification	ED 57% and FPF 55%	[43]
Colistin + Rifampicin	Spray drying	ED 96% and FPF 92%	[44]
Isoniazid + Rifampicin + Pyrazinamide	Spray drying	ED 89% and FPF 46%	[20]
Rifapentine + Moxifloxacin + Pyrazinamide	Spray drying with or without 10% leucine	FPF 64% and 56% with and without leucine, respectively	[21]
Isoniazid + Rifabutin	Spray drying with poly(lactic acid) (L-PLA)	FPF 79%	[49]
Rifampicin	Spray drying and solvent evaporation with PLGA nanoparticles and leucine	FPF 67%	[47]
Rifampicin	Flash frozen with PLGA and mannitol	FPF 54%	[48]
Ciprofloxacin	Spray drying with 50% mannitol	FPF 44%	[50]
Tobramycin	Spray drying with mannitol	ED 97% and FPF 29%	[46]
Azithromycin	Spray drying with mannitol	ED 92% and FPF 38%	[46]
Moxifloxacin HCl	Spray drying with DPPC	ED 86% and FPF 67%	[45]
Ofloxacin	Spray drying with DPPC	ED 97% and FPF 68%	[45]

13.12 Future Considerations

The tensile-strength distribution model has been found to be useful to explain de-agglomeration behaviour of some powders; however, it is a theoretical approach that has been developed based on a number of assumptions and does need to be applied in a broader way to compounds to ensure that it does have universal application. For example, mechanofused powders have been shown to decrease the work of cohesion and tensile strength (as expected) due to the coating of the particles with thin layers of hydrophobic magnesium stearate. However, the packing fraction of mechanofused powders also has been found to increase. The resultant outcome of better aerosolization has resulted from a favourable

balance between lower work of cohesion and increased packing fraction. It can be debated that the equation proposed by Kendall and Stainton [34] does not contain the complexity required for the comparison of two cohesive matrices of materials of different surface characteristics; thus, factors may need to be included in theoretical models to account for surface morphology and chemistry.

De-agglomeration is a complex phenomenon, especially for multi-drug cohesive matrices. It is vitally important to improve our understanding of de-agglomeration mechanisms in order to understand and improve the de-agglomeration capacity of powders, particularly high dose powders where the major mechanism of aerosolization is de-agglomeration. Ongoing efforts need to be made to develop approaches to optimize strategies for better aerosolization.

There are a number of delivery challenges for drugs in the treatment of tuberculosis. First, in order to ensure an effective balance between drug dissolution to produce high local concentrations of drug and drug particle AM uptake to deliver drug to the MTb within the AMs and granulomas, the influence of size, shape and surface of particles on macrophages uptake versus dissolution should be established.

Secondly, an understanding of the drug-deposition patterns of high dose and thus high-density aerosol plumes in the lung and their subsequent implications are important, particularly in understanding the dissolution of drugs. For example, deposition of single or multi-particles of drugs onto the alveolar surface could influence the mechanism and rate of dissolution and thus the drug's distribution, absorption and toxicity in the lung.

Finally, dissolution mechanisms of drugs in the lung are not clear and require further research. For example, the role of lung surfactant on the dissolution of drugs may be a major factor on the wetting of solids, as well as the mechanism and rate of dissolution and retention of drugs in the lung through incorporation in the liquid crystalline nanostructures.

References

[1] Frieden, T.R., Sterling, T.R., Munsiff, S.S. *et al.* (2003) Tuberculosis. *Lancet*, **362**, 887–899.
[2] Hanif, S.N.M. and Garcia-Contreras, L. (2012) Pharmaceutical aerosols for the treatment and prevention of Tuberculosis. *Front. Cell. Infect. Microbiol.*, **2**, Article 118.
[3] World Health Organization (2012) Global tuberculosis report 2012. WHO/HTM/TB/2012.6.
[4] World Health Organization (2012) Definitions and reporting framework for tuberculosis - 2012 revision.
[5] Das, S., Tucker, I. and Stewart, P. (2014) Inhaled Dry Powder Formulations for Treating Tuberculosis. *Curr. Drug Deliv.*, **12**, 26–39.
[6] Cayabyab, M.J., Macovei, L. and Campos-Neto, A. (2012) Current and novel approaches to vaccine development against tuberculosis. *Front. Cell. Infect. Microbiol.*, **2**, Article 154.
[7] Louw, G.E., Warren, R.M., Gey Van Pittius, N.C. *et al.* (2009) A Balancing Act: Efflux/Influx in Mycobacterial Drug Resistance. *Antimicrob. Agents Chemother.*, **53**, 3181–3189.
[8] Gonzalez-Juarrero, M. (2012) Immunity to TB and targets for immunotherapy. *Immunotherapy*, **4**(2), 187–199.
[9] Zhang, D., Lu, Y., Liu, K. *et al.* (2012) Identification of less lipophilic riminophenazine derivatives for the treatment of drug-resistant tuberculosis. *J. Med. Chem.*, **55**, 8409–8417.
[10] Manfredi, R. and Calza, L. (2009) Extensively drug-resistant tuberculosis in patients recently immigrated from Eastern Europe. Microbiological, therapeutic, and public health features. *Clin. Microbiol. Infect.*, **15**, S133.

[11] World Health Organization (2012) Treatment of tuberculosis 2012: Guidelines, 4th edn, WHO/HTM/TB/2009.420.
[12] Adane, A.A., Alene, K.A., Koye, D.N. and Zeleke, B.M. (2013) Non-Adherence to Anti-Tuberculosis Treatment and Determinant Factors among Patients with Tuberculosis in Northwest Ethiopia. *PLOS One*, **8**(11), e78791.
[13] Jawahar, N. and Reddy, G. (2012) Nanoparticles: A novel pulmonary drug delivery system for tuberculosis. *J. Pharm. Sci. Res.*, **4**, 1901–1906.
[14] Taylor, G. and Gumbleton, M. (2004) Aerosols for macromolecule delivery: Design challenges and solutions. *Am. J. Drug Deliv.*, **2**, 143–155.
[15] Pandey, R. and Khuller, G.K. (2005) Antitubercular inhaled therapy: opportunities, progress and challenges. *J. Antimicrob. Chemother.*, **55**, 430–435.
[16] Lee, S.H., Teo, J., Heng, D. *et al*. (2013) Synergistic Combination dry powders for inhaled antimicrobial therapy: Formulation, characterization and in vitro evaluation. *Eur. J. Pharm.*, **83**, 275–284.
[17] Dolovich, M.B and Dhand, R. (2011) Aerosol drug delivery: developments in device design and clinical use. *Lancet*, **377**, 1032–1045.
[18] Sou, T., Meeusen, E.N., de Veer, M. *et al.* (2011) New development in dry powder pulmonary vaccine delivery. *Trends Biotechnol.*, **29** 191–198.
[19] Misra, A., Hickey, A.J., Rossi, C. *et al.* (2011) Inhaled drug therapy for treatment of tuberculosis. *Tuberculosis*, **91**, 71–81.
[20] Chan, J., Chan, H.K., Prestidge, C. *et al*. (2013) A novel dry powder inhalable formulation incorporating three first-line anti-tubercular antibiotics. *Eur. J. Pharm. Biopharm.*, **83**, 285–292.
[21] Chan, J., Tyne, A., Pang, A. *et al.* (2014) A Rifapentine-Containing Inhaled Triple Antibiotic Formulation for Rapid Treatment of Tubercular Infection. *Pharm. Res.*, **31**, 1239–1253.
[22] Zhou, Q.T., Qu, L., Larson, I. *et al.* (2010) Improving aerosolization of drug powders by reducing powder intrinsic cohesion via a mechanical dry coating approach. *Int. J. Pharm.*, **394**, 50–59.
[23] El-Sherbiny, I.M., Villanueva, D.G., Herrera and Smyth, H.D.C. (2011) in Controlled Pulmonary Drug Delivery (eds H.D.C. Smyth and A. Hickey), Springer, Dordrecht, pp. 101–126.
[24] Williams, M.C. (1977) Conversion of lamellar body membranes into tubular myelin in alveoli of fetal rat lungs. *J. Cell Biol.*, **72**, 260–277.
[25] Haagsman, H.P. and Golde, L.M. (1991) Synthesis and assembly of lung surfactant. *Annu. Rev. Physiol.*, **53**, 441–464.
[26] Gil, J. and Reiss, O.K. (1973) Isolation and characterization of lamellar bodies and tubular myelin from rat lung homogenates. *J. Cell Biol.*, **58**, 152–171.
[27] Epand, R.M. (1998) Lipid polymorphism and protein–lipid interactions. *Biochim. Biophys. Acta*, **1376**, 353–368.
[28] Stewart, P. (1986) Particle interaction in pharmaceutical systems. *Pharm. Int.*, **7**, 146–149.
[29] Das, S., Behara, S., Bulitta, J. *et al*. (2012) Powder Strength Distributions for Understanding De-agglomeration of Lactose Powders. *Pharm. Res.*, **29**, 2926–2935.
[30] Tsubaki, J. and Jimbo, G. (1984) Theoretical analysis of the tensile strength of a power bed. *Powder Technol.*, **37**, 219–227.
[31] Nikolakakis, I. and Pilpel, N. (1985) Effect of particle shape on the tensile strength of powders. *Powder Technol.*, **42**, 279–284.
[32] Nikolakakis, I. and Pilpel, N. (1988) Effect of particle size and particle shape on the tensile strength of powders. *Powder Technol.*, **56**, 95–103.
[33] Weiler, C., Wolkenhauer, M., Trunk, M. and Langguth, P. (2010) New model describing the total dispersion of dry powder agglomerates. *Powder Technol.*, **203**, 248–253.
[34] Kendall, K. and Stainton, C. (2001) Adhesion and aggregation of fine particles. *Powder Technol.*, **121**, 223–229.
[35] Cross, M. and Das, S. (2014) University of Otago [A New Zealand Pharmacy Education and Research Foundation-funded Research Project Report (grant no. 254a) submitted to the School of Pharmacy, University of Otago, 7th March 2014].

[36] Das, S.C. (2014) in *D4 – Devices for Diagnostics and Drug Delivery Conference* (Dunedin, University of Otago, New Zealand, 26–27 November 2014).
[37] Zhou, Q., Gengenbach, T., Denman, J.A. *et al.* (2014) Synergistic Antibiotic Combination Powders of Colistin and Rifampicin Provide High Aerosolization Efficiency and Moisture Protection. *AAPS J.*, **16**, 37–47.
[38] Das, S.C., Behara, S.R.B., Larson, I. *et al.* (2013) Influence of intermediate cohesive fraction of lactose on lactose powder aerosolisation behaviour determined by powder agglomerate strength distributions. *J. Aerosol Med. Pulmon. Drug Deliv.*, **26**, A4.
[39] Das, S.C., Behara, S.R.B., Morton, D.A.V. *et al*. (2013) Importance of particle size and shape on the tensile strength distribution and de-agglomeration of cohesive powders. *Powder Technol.*, **249**, 297–303.
[40] Chan, J., Duke, C., Ong, H.X. *et al*. (2014) A Novel Inhalable Form of Rifapentine. *J. Pharm. Sci.*, **103**, 1411–1421.
[41] Young, P.M., Crapper, J., Philips, G. *et al*. (2014) Overcoming Dose Limitations Using the Orbital® Multi-Breath Dry Powder Inhaler. *J. Aerosol Med. Pulmon. Drug Deliv.*, **27**, 138–147.
[42] Son, Y. and McConville, J.T. (2011) A new respirable form of rifampicin. *Eur. J. Pharm. Biopharm.*, **78**, 366–376.
[43] Parikh, R., Dalwadi, S., Aboti, P. and Patel, L. (2014) Inhaled microparticles of antitubercular antibiotic for in vitro and in vivo alveolar macrophage targeting and activation of phagocytosis. *J. Antibiot.*, **67**(5), 387–394.
[44] Zhou, Q., Morton, D., Yu, H. *et al*. (2013) Colistin Powders with High Aerosolisation Efficiency for Respiratory Infection: Preparation and In Vitro Evaluation. *J. Pharm. Sci.*, **102**, 3736–3747.
[45] Duan, J., Vogt, F.G., Li, X. *et al*. (2013) Design, characterization, and aerosolization of organic solution advanced spray-dried moxifloxacin and ofloxacin dipalmitoylphosphatidylcholine (DPPC) microparticulate/nanoparticulate powders for pulmonary inhalation aerosol delivery. *Int. J. Nanomed.*, **8**, 3489–3505.
[46] Li, X., Vogt, F.G., Hayes, D. *et al*. (2014) Design, Characterization, and Aerosol Dispersion Performance Modeling of Advanced Co-Spray Dried Antibiotics with Mannitol as Respirable Micro particles/Nanoparticles for Targeted Pulmonary Delivery as Dry Powder Inhalers. *Pharm. Drug Deliv. Pharm. Technol.*, **103**, 2937–2949.
[47] Sung, J.C., Padilla, D.J., Garcia-Contreras, L. *et al*. (2009) Formulation and Pharmacokinetics of Self-Assembled Rifampicin Nanoparticle Systems for Pulmonary Delivery. *Pharm. Res.*, **26**, 1847–1855.
[48] Lawlor, C., O'Sullivan, M.P., Rice, B. *et al*. (2012) Therapeutic aerosol bioengineering of targeted, inhaled microparticle formulations to treat Mycobacterium tuberculosis (MTb). *J. Mater. Sci. Mater. Med.*, **23**, 89–98.
[49] Muttil, P., Kaur, J., Kumar, K. *et al*. (2007) Inhalable microparticles containing large payload of anti-tuberculosis drugs. *Eur. J. Pharm. Sci.*, **32**, 140–150.
[50] Adi, H., Young, P.M., Chan, H.-K. *et al*. (2010) Co-spray-dried mannitol-ciprofloxacin dry powder inhaler formulation for cystic fibrosis and chronic obstructive pulmonary disease. *Eur. J. Pharm. Sci.*, **40**, 239–247.
[51] Anonymous (2013) Tobramycin inhalation powder (Tobi Podhaler) for cystic fibrosis. *Med. Lett. Drugs Ther.*, **55**, 51–52.
[52] de Boer, A.H., Hagedoorn, P., Westerman, E.M. *et al*. (2006) Design and in vitro performance testing of multiple air classifier technology in a new disposable inhaler concept (Twincer®) for high powder doses. *Eur. J. Pharm. Sci.*, **28**, 171–178.
[53] de Boer, A.H., Hagedoorn, P., Woolhouse, R. and Wynn, E. (2012) Computational fluid dynamics (CFD) assisted performance evaluation of the Twincer (TM) disposable high-dose dry powder inhaler. *J. Pharm. Pharmacol.*, **64**, 1316–1325.
[54] Friebel, C. and Steckel, H. (2010) Single-use disposable dry powder inhalers for pulmonary drug delivery. *Expert Opin. Drug Deliv.*, **7**, 1359–1372.
[55] Schuster, A., Haliburn, C., Döring, G. *et al*. (2013) Safety, efficacy and convenience of colistimethate sodium dry powder for inhalation (Colobreathe DPI) in patients with cystic fibrosis: a randomised study. *Thorax*, **68**, 344–350.

Section 4

Alternative Approaches

14

Respirable Bacteriophage Aerosols for the Prevention and Treatment of Tuberculosis

Graham F. Hatfull[1] and Reinhard Vehring[2]

[1] Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA
[2] Department of Mechanical Engineering, University of Alberta, Edmonton, AB, Canada

14.1 Introduction

In this chapter we will discuss the general question of if and how respirable bacteriophages could be used therapeutically for tuberculosis infections. These concepts remain largely hypothetical in that there are few animal studies and no human trials to our knowledge that have addressed the potential efficacy of phage therapy for human tuberculosis. Nonetheless, with the ongoing accumulation of clinically prevalent antibiotic-resistant strains of *Mycobacterium tuberculosis* and the difficulties encountered in treating patients with multidrug- and extensively drug-resistant strains, alternative therapies warrant consideration. Here we review basic aspects of bacteriophages, consider the availability and features of mycobacteriophages with therapeutic potential, and consider ways in which these phages might be manufactured and delivered as an inhaled pharmaceutical aerosol.

14.1.1 Bacteriophages

Bacteriophages are viruses that infect bacterial hosts. They are near ubiquitous and phages have been identified for most bacterial hosts for which they have been looked for. Their host-range – the variety of bacterial hosts they productively infect – differs for each phage

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

isolate, but is typically narrow, sometimes constrained to one or a few strains within a bacterial species [1] Others have broader host-ranges and may infect more than one host species within the same genus. It is relatively unusual for phages to infect hosts within more than one genus. This typically occurs only when the genera are very closely related. Here we will discuss the mycobacteriophages, which are bacteriophages that infect hosts such as *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. Some mycobacteriophages infect only some substrains of *M. smegmatis*, whereas others infect both *M. smegmatis* and *M. tuberculosis* [2, 3]. None of these mycobacteriophages are known to infect hosts in other bacterial genera [4].

Bacteriophages are typically rather simple compared with their hosts. There are a variety of different virion morphologies, but the *Caudovirales* are the most abundant type, with an icosahedral capsid (head) containing the double-stranded DNA (dsDNA) genome and a tail (Figure 14.1).

There are three main morphological families within this order, the *Siphoviridae*, the *Myoviridae*, and the *Podoviridae*, which differ in their tail type: long flexible non-contractile, contractile, and short stubby tails, respectively. All of the mycobacteriophages are in these first two subtypes [5]. Host specificity is conferred largely by the components at the tip of the tail, which are responsible for adsorption to the host and host recognition, which must occur prior to the irreversible decision to trigger DNA infection [3]. Once activated, DNA passes out of the capsid, through the tail, through the host wall and membrane and into the cytoplasm of the bacterium; the protein coat remains on the outside of the cell and serves no further purpose in phage replication.

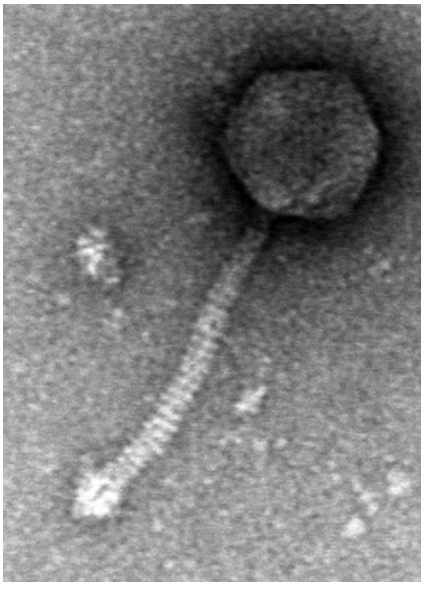


Figure 14.1 *Electron micrograph of mycobacteriophage D29. Phage D29 has a head 55 nm wide and a flexible non-contractile tail 130 nm long. It is typical of phages with siphoviral morphologies that are common among the mycobacteriophages*

The plaque assay is a common method used for *de novo* phage isolation, for quantifying phage lysates, determining host-range, and gaining insights into viral life cycles. When a phage sample is mixed with host cells, plated on solid media with a soft agar overlay, and incubated, visible plaques are observed on the bacterial lawn where a single phage particle infected a host cell and then amplified through a series of infections until the bacterial lawn stopped growing (Figure 14.2a). Because each plaque is derived from a single particle, the number of plaques, the dilution factor, and the amount spotted enables simple calculation of the concentration of the initial phage sample (i.e., its titer), which can be expressed as plaque-forming units per milliliter (pfu/ml). The total number of particles in a plaque varies substantially, but can be up to 5×10^8 pfu [6], reflecting multiple rounds of replication where the burst size (the average number of particles released for each infected cell) is typically 30–200 pfu. A related assay is a phage spot test in which a soft agar overlay containing bacteria is prepared on solid media, onto which 5–10 µl phage samples are added, allowed to dry, and then incubated until the bacterial lawn is formed (Figure 14.2b). This is convenient as many different spots can be added to a single Petri plate, and the titer of a lysate can be determined by spotting serial dilutions that span a wide range of phage concentrations. This is an easy way to examine the plaque morphotypes as the infected areas of the spots can be easier to evaluate than can individual plaques. In Figure 14.2b the turbid plaque morphotype (top row) associated with a temperate life style (see below) is easily distinguishable from clear plaque morphotypes (second and third rows, Figure 14.2b) of lytic phages. The phage in the bottom row of Figure 14.2b forms plaques that are intermediate between the two extreme phenotypes and may form lysogens at low frequency (see below).

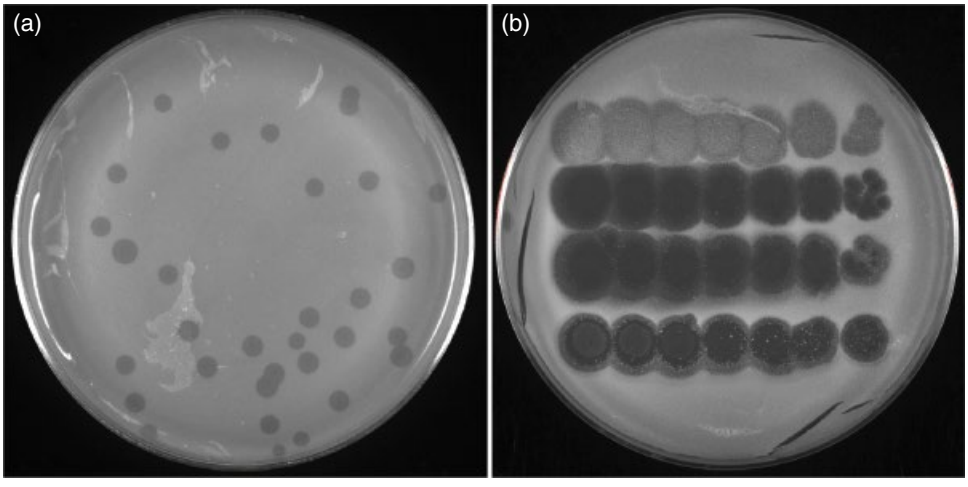


Figure 14.2 *(a) A plaque assay showing individual phage plaques growing on a lawn of M. smegmatis. (b) A phage spot test in which four different phage samples were serially diluted 10-fold and 5 µl of each dilution spotted onto a lawn of M. smegmatis (with most concentrated to least concentrated going left to right). The phage in the top row is temperate and forms turbid plaques; the phages in the second and third rows are clear and grow lytically; the phage in the fourth row forms lightly turbid plaques and may form lysogens at low frequency*

Bacteriophages can be broadly classified as being either lytic or temperate. Lytic phages usually pursue a single pathway once the DNA is inside the cell. Early genes are expressed and some of these may participate with host enzymes to replicate phage DNA. There are numerous different ways in which they do so; for example, some phages encode their own DNA polymerase, whereas others utilize a host polymerase [7]. Late genes (typically 20–25) are then expressed, which are primarily involved in virion structure and assembly, and capsid and tail substructures are assembled. The newly replicated genomes are then packaged into the heads and the tails attached to form mature particles. The cell then lyses to release the progeny phage.

Temperate phages are fully capable of lytic growth, but have access to a different outcome of infection. Once the DNA is inside the cell, an alternative pathway (lysogeny) can be chosen, in which early expression and accumulation of a repressor protein shuts down lytic gene expression. Usually (but not always), the phage DNA is integrated site-specifically into the host chromosome, and constant repressor expression is required for maintenance of the lysogenic state. Lysogenic bacteria are stable but integrated prophages are capable of induction into lytic growth, either spontaneously or in response to environmental changes (such as ultraviolet radiation). Temperate phages can be distinguished from lytic phages in that they form turbid rather than clear plaques (Figure 14.2). Phages forming clear plaques undergo only lytic growth resulting in phage amplification and cell death, whereas temperate phages form turbid plaques in which a subset of cells form lysogens which continue to grow, while a majority of infected cells undergo lytic growth. The proportion of temperate phage infections that yield lysogeny varies among phages and with environmental conditions [including multiplicity of infection (m.o.i.), the average number of infecting viral particles per host cell], but is typically 5–20% of the cells. Lysogens can be readily identified by their immunity to superinfection by the same phage, and their ability to spontaneously release phage particles into the culture supernatant. Genomic analysis shows that most sequenced bacterial genomes contain one or more (and sometimes many) prophages.

14.1.2 Mycobacteriophages

A large number of mycobacteriophages have been isolated using *M. smegmatis* as a host (http://phagesdb.org), in addition to those phages isolated previously for strain-typing purposes. Complete genome sequences have been determined for about 800 of these, revealing substantial genetic diversity. Using nucleotide-sequence similarity and gene-content profiles these phages can be grouped into about 20 clusters of related genomes, and nine singletons that have no close relatives. Many of the clusters encompass substantial diversity and can be divided further into subclusters [4]. These phage genomes have provided numerous insights into phage diversity and viral evolution, gene regulation, novel gene functions, and tools for tuberculosis genetics (for recent reviews see [4, 5, 8]). We will focus here on aspects relevant to the control of tuberculosis.

There is a strong correlation between the ability of these phages to infect *M. tuberculosis* and their genomic type. For example, there are phages within all five Cluster K subclusters (including TM4) [3, 9] and in subclusters A2 and A3 (including phages L5 and D29) [3, 10, 11] that infect *M. tuberculosis* efficiently. Other mycobacteriophages either don't infect *M. tuberculosis* or do so at very low plating efficiencies (i.e., the phage titer is substantially

lower than it is on *M. smegmatis*). It is notable that most phages within Cluster G do not efficiently infect *M. tuberculosis*; however, mutants, detected at a frequency of about 10^{-5}, can efficiently infect *M. tuberculosis* [12]. When isolated and purified, these mutants infect *M. smegmatis* and *M. tuberculosis* with equal efficiencies. Their mutations are within a putative tail fiber gene [3].

Interestingly, phages of Clusters A2, A3, K and G are temperate or are likely recent lytic derivatives of temperate parents, a relevant feature as lytic phages are anticipated to be the most useful in therapeutic applications. Phage D29 (subcluster A2), for example, is one of the most lytic phages against both *M. tuberculosis* and *M. smegmatis,* and is a derivative of a temperate parent due to a large deletion removing the repressor gene [11]. It retains a functional integration system [13] and can form lysogens in the presence of the repressor of the related phage, L5 [11, 14]. Another commonly used phage, TM4, is a subcluster K2 isolate that has lost both its repressor and its integration functions [9], although it does not kill its host as efficiently as does D29. Genetic manipulation of another Cluster K phage, Adephagia, by targeted removal of its integrase and repressor genes, generates a derivative that kills both *M. tuberculosis* and *M. smegmatis* as efficiently as does D29 (Zaritza Petrova, Gregory Broussard and G.F. Hatfull, unpublished observations). Cluster G phages form lysogens at relatively low efficiencies and employ a novel integration-dependent immunity system [15, 16], but lytic variants capable of infecting *M. tuberculosis* have been isolated (Zaritza Petrova, Gregory Broussard and G.F. Hatfull, unpublished observations). Finally, phage DS6A is the only phage that is known to be specific for infection of *M. tuberculosis* complex strains and which does not infect *M. smegmatis* [17, 18]. It does not appear to form stable lysogens, but does encode an integration system [19].

Rather little is known about why mycobacteriophages of other genomic types do not infect *M. tuberculosis*. A likely explanation for many phages is the lack of a suitable receptor, and it has been shown that although phage Giles cannot infect *M. tuberculosis*, it can convert electroporated DNA into particles that can then be propagated on *M. smegmatis* [6]. Other possible explanations are the presence of restriction-modification systems, or Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems. However, there is no evidence for an active restriction-modification system in *M. tuberculosis*, and although *M. tuberculosis* contains CRISPR-like sequences, they lack any close similarity to known mycobacteriophage sequences that would be required to confer protection from infection. Understanding the determinants of host-range is likely to be helpful in engineering phages so that they can efficiently infect and kill *M. tuberculosis.*

Phage resistance is an important concern in any therapeutic application, and little is also known about mechanisms of resistance to phage infection of *M. tuberculosis*. Spontaneous D29-resistant mutants of *M. smegmatis* can be readily isolated, appearing as small colonies on solid media seeded with phage particles. However, they grow poorly in the presence of D29 and are genetically unstable, losing resistance after being grown in the absence of phage. Interestingly, overexpression of a wild-type chromosomal gene, *mpr*, confers high levels of D29 resistance apparently by interference of productive DNA injection [20, 21]. Overexpression of *mpr* confers resistance specifically for D29 and its A2 subcluster relative L5, but not to Bxb1 (subcluster A1), TM4 (K2), or I3 (C). Mutants resistant to Cluster G and K phages have been isolated but are not yet well characterized, and cross-resistance patterns have yet to be determined.

14.1.3 *Mycobacterium tuberculosis* as a Host for Phage Infection *in vivo*

Genome sequences of a broad array of mycobacterial species have been sequenced along with many individual strains of *M. tuberculosis*. Several of these (e.g., *M. ulcerans, M. avium*) clearly harbour full-length intact prophages, but not *M. tuberculosis*. When *M. tuberculosis* H37Rv was first sequenced no full-length prophages were identified, although it contains two ~10 kbp prophage-like elements, ϕRv1 and ϕRv2 [22, 23]; most other *M. tuberculosis* strains contain either one or both of these. Although they encode phage functions such as a capsid and an active integrase [24, 25], particles containing their DNA have not been described and they are not known to play any role in virulence.

M. tuberculosis lives in very restricted environments such as in macrophages and in granulomas. There is no known reservoir outside humans, and thus the question arises as to whether *M. tuberculosis* is exposed to phages in the natural environment. A couple of lines of evidence suggest that it is, or at least has been until recently in its evolutionary history. First, *M. tuberculosis* strains have considerable variation among the CRISPR spacers (making them suitable for strain identification by spoligotyping [26]). CRISPRs play well-defined roles in preventing introduction of foreign DNA, including protection from phage infection. Second, *M. tuberculosis* contains an unusually large number of toxin–antitoxin (TA) systems. TA systems have been implemented in phage resistance through abortive infection [27, 28]. Therefore, it is plausible that they serve similar functions in *M. tuberculosis*.

14.1.4 Mycobacteriophages and TB Diagnosis

Mycobacteriophages have proven useful in several potential applications for tuberculosis (TB) diagnosis and drug-susceptibility testing. One application is phage typing, which was explored extensively beginning in the 1950s. In phage typing, panels of phages are identified with specific host-ranges that enable identification of *M. tuberculosis* [29]. A second application is the phage-amplification assay [30], which has been commercialized and extensively evaluated as the FASTPlaqueTB assay [31, 32]. A third application is the use of recombinant reporter mycobacteriophages. In this approach, phages carrying a reporter gene such as firefly luciferase or a fluorescent protein gene infect a clinical specimen that can be readily monitored, allowing drug susceptibilities to be determined empirically [33–35]. Clinical evaluation shows considerable promise for these diagnostic strategies [36, 37].

14.2 Treatment or Prevention of Tuberculosis Using Phage-based Agents

14.2.1 Bacteriophages as Therapeutic Agents

The concept of employing phages therapeutically as antimicrobial agents is certainly not a new one, and emerged from the pioneering work of d'Herelle almost a hundred years ago [38]. Major phage therapy programmes were developed in the former Soviet states and Poland, often with the view of developing patient-specific therapies targeted towards a specific pathogenic strain. There has been a much renewed interest in these applications in western countries, and a variety of phage applications have been developed and approved for use, including phage cocktails for controlling *Escherichia coli* and *Listeria* infections

in meat products [39]. Numerous human therapeutic trials have been initiated or proposed and hopefully outcomes and approval will be resolved in the coming years. However, Food and Drug Administration (FDA) approval within the US requires consideration of numerous facets not encountered with approval of standard antibiotics. First, if viable phage particles are used therapeutically, then growth and amplification of phage numbers is expected, and while this ought to be therapeutically beneficial, it incorporates the possibility that mutant derivatives can arise that would have different – and unpredictable – properties from the 'approved' starting material. These issues are not dissimilar to those arising with the use of live attenuated viral vaccines, such as Influenza virus vaccines. Second, phage resistance is to be anticipated, although it can be countered by the use of phage cocktails in which the constituent phages do not share resistant mechanisms – i.e., selection for resistance to one phage in the cocktail does not give rise to resistance to other phages in the cocktail. Third, many phages are known to encode toxins and other potentially harmful products, as well as many genes of unknown (and thus potentially harmful) functions. Finally, there is the potential concern of endotoxins present in the host being released as a consequence of phage-mediated lysis, giving rise to a Jarisch–Herxheimer reaction [40]. These issues, along with validation of routes of infection, dosage, and pharmacokinetics, necessarily require substantial investment into a potential new phage drug, notwithstanding the apparent simplicity, presumed low toxicity, and general abundance of bacteriophages.

14.2.2 Prospects for Using Mycobacteriophages for Therapy or TB Prevention

The increase in prevalence of multi-drug-resistant (MDR) and extensively drug-resistant (XDR) strains necessitates the discovery of new antibiotics and consideration of alternative therapies. The therapeutic use of phages has been postulated, but their prospects may be brighter for prophylaxis than for treatment [41]. Prophylactic interruption of transmission is attractive in that inhaled bacteria should be accessible to the phage and only small numbers of bacteria need to be eliminated. Thus, a sufficiently high multiplicity of infection (m.o.i.) should be achievable. Moreover, phage resistance ought to be rare due to the low quantity of bacteria present. Phage treatment of an existing infection is complicated by access concerns, as many of the bacilli are within macrophages or walled off in granulomas. Nonetheless, it is plausible that a phage application in the late stages of infection, where there are substantial extracellular bacteria, could help to extend the effectiveness and timeframe of other treatments. Unfortunately, there are few suitable model organisms for evaluating this potential, although one study has demonstrated the effectiveness of phages in treating guinea pigs [42]. We note that combinations of phages and antibiotics have been tested for other infections such as by *Pseudomonas aeruginosa* [43].

A plausible approach to optimize phage delivery to *in vivo* mycobacterial targets is to use a surrogate host for delivery [44]. For example, application of *M. smegmatis* cells infected with phage TM4 to macrophages infected with *M. tuberculosis* resulted in significant reductions in intracellular bacteria. The clinical applicability of such an approach has yet to be evaluated. A related approach might be to use an inducible lysogen that enters lytic growth in an infected macrophage. If such as phage could be engineered then it would simplify delivery to the site of infection, and operate such as to induce lytic growth in infected macrophages, leading to death of the lysogen and release of large numbers of phage particles that would then infect and kill any residue mycobacterial cells. In such

scenarios it is critical that the surrogate bacterial host does not cause disease or any adverse host responses.

Phage components may also have potential for TB therapy. They lack the ability to replicate and amplify during therapy, which may negatively impact efficacy while simplifying regulatory concerns. Following advances in other infectious diseases, there are two obvious approaches. One is the development of pyocins – naturally occurring phage tail-like structures with antimicrobial activity – with the capability of infecting *M. tuberculosis*. These have been developed for *Clostridium difficile* and *Escherichia coli* [45, 46] and could plausibly be developed for *M. tuberculosis,* taking advantage of the considerable mycobacteriophage genome-sequence information available. The second is the potential to use phage-encoded lysis proteins (endolysins) to promote killing by exogenous addition, as demonstrated for pneumococcal, streptococcal and *Bacillus anthracis* infections [47–50]. Although mycobacteriophages encode similar endolysins that cleave peptidoglycan [51, 52], their utility in exogenous addition is questionable because of the mycobacterial outer membrane protecting the peptidoglycan. The mycobacteriophages are unusual in encoding a second lysis protein (Lysin B) that cleaves the linkage of the mycolic acid outer later to the cell wall and may have therapeutic potential [53, 54].

14.3 Selection of Mycobacteriophages

What mycobacteriophages might be used for therapeutic or prophylactic applications? As noted above, a large collection of sequenced mycobacteriophages are available, although only some of these infect *M. tuberculosis*. Moreover, these belong to just a few genomic types, each of which is anticipated to use similar adsorption and infection mechanisms and thus be subject to similar resistance mechanisms. Whether developing a therapeutic or prophylactic application, it is desirable to use cocktails of at least three different phages for which cross-resistance is not common. Plausible candidates are D29 (Cluster A2), a lytic mutant of Adephagia (Cluster K), DS6A, and perhaps a Cluster G phage such as BPs, although their cross-resistance patterns are not yet established. With the exception of DS6A all of these phages can be propagated on *M. smegmatis*, avoiding the slow growth and pathogenicity of *M. tuberculosis*. Although the narrow host range of DS6A, which is restricted to *M. tuberculosis*-complex hosts, makes it attractive from a therapeutic perspective, it also severely complicates its laboratory propagation for clinical use.

Additional phages could likely be isolated using *M. tuberculosis* as a host, and screening patient samples. This is not simple, however, because of the slow growth of *M. tuberculosis* and the challenges in screening large number of patient samples. An alternative approach is to isolate and characterize phages of other mycobacterial hosts, in the expectation that clusters of phages related but distinct from those infecting *M. smegmatis* will be recovered, of which a new subset may infect *M. tuberculosis*. Regardless of the approach used, additional phages are clearly needed to construct cocktails with desirable efficacy for which emerging resistance is not an impediment.

A further question is whether to use naturally occurring isolates, or engineered derivatives that have advantageous features. Genetic manipulation of mycobacteriophages can be accomplished either using a shuttle phasmid strategy [55] using chimeric vectors that replicate as plasmids in *Escherichia coli* and as phages in mycobacteria, or by Bacteriophage

Recombineering of Electroporated DNA (BRED) [56, 57], such that constructing recombinant derivatives of many phages is reasonably simple; others may be intractable to these methods due to genome size. The number of different types of recombinants that could be constructed is large, and includes the possibility of introducing genes that modulate the host response, or which alter antibiotic-resistance profiles. In practice, the most direct route towards an approved therapy for TB would likely involve naturally occurring phages, with examination of recombinants as a subsequent developmental stage.

Little is known about the suitability of extant phages for rapid, simple, and inexpensive propagation coupled with reliable stability during storage and use, as well as safety. Most mycobacteriophages can be readily amplified using solid media with a simple composition, but not all phages amplify efficiently in liquid media under standard conditions. Liquid growth is, however, the most attractive method for propagating large quantities of phages for clinical evaluation, and thus warrants further evaluation and optimization for therapeutic candidates. Anecdotally, stability is also somewhat phage dependent, with some phage lysates suffering little or no loss of titer when stored at 4 °C, and others suffering dramatic losses in titer after storage for only relatively short periods of time. Addition of divalent cations (Mg^{2+} and Ca^{2+} in particular), gelatin, and other additives may improve stability when stored at 4 °C, and phage stocks can be stored for long periods if frozen in the presence of a protectorant [such as dimethyl sulfoxide (DMSO)].

14.4 Respiratory Drug Delivery of Phages

Tuberculosis remains in a latent, intracellular state in the majority of infected individuals. Latent tuberculosis is underdiagnosed and access to the pathogen requires transport of the therapeutic agent across a cell wall. From a standpoint of long-term global disease control it is also worthwhile to reduce TB prevalence by preventing transmission. Tubercle bacilli are transmitted mainly via the aerosol route from an individual with active pulmonary tuberculosis, causing a primary infection within the lungs of the exposed individual [58]. While tuberculosis in general is a multisystemic disease with extrapulmonary manifestations, both the origin and target of infecting bacilli are located in the lungs. Hence, it may be advantageous to control transmission by respiratory delivery of mycobacteriophages, with the intent to render patients with active TB non-contagious or reduce susceptibility to infection in a prophylactic mode, especially in cases where traditional treatment options fail due to drug resistance.

To our knowledge, mycobacteriophage therapy in humans has not been reported and *in vivo* studies in rodents have produced mixed results [59, 60]. A factor in the limited success of *in-vivo* work may be the inability of the phages, which were administered by injection [44, 61], to reach intracellular bacteria in sufficient numbers. Work related to inhalation of mycobacteriophages is very limited: Liu *et al*. aerosolized phage D29 using a six-jet Collison nebulizer and collected viable phages from the aerosol in various collection media [62], albeit with a severe 2 to 3 $\log_{10}$ loss relative to the initial titer in the phage suspension [63]. Hence, a discussion on the feasibility of delivering mycobacteriophages by inhalation must be based on existing work utlizing similar phages. Known mycobacteriophages belong to the order C*audovirales*, i.e., they are tailed phages with dsDNA genomes, and are either myoviruses or siphoviruses [41].

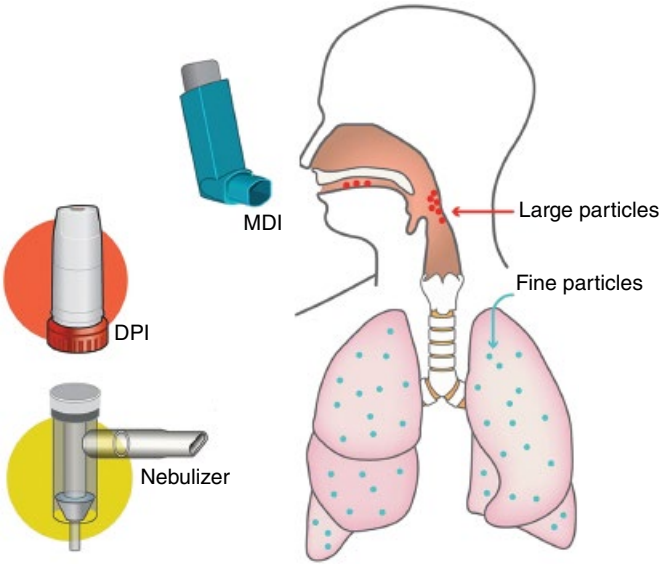


Figure 14.3 *Device options for respiratory delivery of bacteriophages*

Options for phage delivery include nebulizers, dry-powder inhalers (DPIs), and potentially pressurized metered-dose inhalers (MDIs) as shown in Figure 14.3.

Nebulizers have been a popular choice for early phage-inhalation studies because they require only limited formulation development and can provide a fine aerosol suitable for efficient delivery to the lung at low inhalation flow rates. Most nebulizer types can aerosolize suspensions and accept phage preparations without much further preparation. Golshahi *et al*. successfully aerosolized a *Burkholderia cepacia* specific *Myoviridae* phage, KS4-M, using a compressor-driven air jet nebulizer or, alternatively, a vibrating mesh nebulizer [64]. The results of this *in-vitro* study demonstrated that this phage could be nebulized with less than 1 $\log_{10}$ titer loss, regardless of the nebulizer used. Both devices were capable of delivering a high lung dose on the order of 10^7 pfu within a few minutes of nebulization time. Recently this group demonstrated efficacy of respiratory phage therapy in *Burkholderia cepacia* complex infections in a mouse model, delivering several *Myoviridae* phages to the animals via a nose-only inhalation device in combination with a jet nebulizer [65]. This study also showed a clear advantage of respiratory phage delivery over administration by an intraperitoneal route. While these results are encouraging, alternatives to nebulizers should be considered, because nebulizers are comparatively expensive, require considerable training for care-givers and patients, or need a developed infrastructure with access to electricity or suitable supplies for cleaning and disinfection. For tuberculosis which affects primarily patients in resource-poor settings, simpler inhalation devices are preferable.

There are several options for processing phages into a dry powder suitable for delivery from a DPI. Lyophilization is a standard technique for conversion of biologics, including phages [66], into dry forms. It is a recommended method for the long-term preservation of phage stock [67] and storage stability (less than 1 $\log_{10}$ titer loss over 2.5 years) of many

freeze-dried mycobacteriophages has been demonstrated [68]. In a systematic study [69] on several stabilizing excipients, sucrose and trehalose were found to be most successful in preserving *Myoviridae* phage Interspaced Short Palindromes during freeze drying with a combined processing loss and loss on storage (for 37 months at 4 °C) of less than 1 $\log_{10}$. Before it can be used in a DPI the freeze-dried material must be micronized to a respirable particle size. Golshahi *et al*. lyophilized phage KS4-M and a giant *Myoviridae* phage specific to *Pseudomonas aeruginosa*, ΦKZ, with lactose and lactoferrin, reporting a titer loss upon processing of 2 $\log_{10}$ and 1 $\log_{10}$, respectively [70]. Subsequent milling in a mixer mill did not damage the dried phages. *In vitro* lung doses of 10^6 to 10^7 pfu were achieved with an inexpensive, capsule-based DPI (Aerolizer®) at an inhalation flow rate of 60 l/min [70].

A second option for processing phages into dry powders has been investigated: spray drying. *Myoviridae* phages KS4-M, KS14 and ΦKZ were atomized using a vibrating mesh atomizer and then dried in a process that was optimized for low thermal stress [71]. Individual phages and also phage cocktails were formulated with the stabilizing excipients trehalose, and optionally casein sodium salt or surfactants, and leucine as a dispersibility enhancer. Phages were processed with low titer loss (0.4–0.8 $\log_{10}$) into a highly respirable powder whose aerosol performance when delivered from a simple dry-powder inhaler (Aerolizer®) was better than that of most commercial inhalation products. *In vitro* lung doses between 10^7 to 10^8 pfu were achieved, matching or exceeding that of nebulizer delivery. Vandenheuvel *et al.* confirmed that trehalose is effective as a desiccoprotectant for phages and also demonstrated the feasibility of spray drying phages with a twin-fluid atomizer [72]. However, those authors found markedly lower process loss for a *Podoviridae* phage (LUZ19) compared with *Pseudomonas* phage Romulus, a myovirus, indicating that manufacturability may be phage specific. In a subsequent study those authors investigated storage stability of phages spray-dried with trehalose as a glass stabilizer. They found an acceptable titer loss after one year in refrigerated storage [73]. Their results on room temperature stability are inconclusive, because the stability study was apparently performed without moisture protection, which may cause over-drying for storage at dry conditions and excessive moisture uptake and subsequent crystallization of the stabilizer upon exposure even to moderate relative humidity. Both effects are known to have a detrimental effect on glass-stabilized biologics. In summary, first studies on the feasibility of spray drying bacteriophages have yielded promising results, but more work, especially with mycobacteriophages, is necessary to find suitable processes and formulations.

MDIs have several characteristics that make them very attractive candidates for use in tuberculosis intervention. MDIs can hold several hundred individual doses in a single canister and are low-cost devices, reducing the device cost for a single administration to less than 10 cents for a generic product. MDIs also have excellent environmental protection and can be used by minimally trained personnel. MDIs are compatible with highly potent drugs with therapeutic doses of less than 1 mg. In this context it is instructive to convert a typical phage lung dose of 10^8 pfu to an equivalent drug mass: assuming that a single phage has a mass on the order of a few femtograms, it follows that the infectious phage mass required for delivery is only of the order of 0.1 µg. Hence, phage therapeutics are highly potent and, therefore, are well suited to the dose-range capabilities of MDIs.

Experience with phages in MDIs is very limited, but studies on related biological material can provide indirect evidence for the feasibility of phage delivery from propellant-based delivery devices. DNA formulated into chitosan nanoparticles did not lose its

transfection efficiency after six weeks of storage in propellant HFA 227ea at room temperature [74]. Freeze-dried, DNA-loaded microemulsions were dispersed in an HFA 134a–ethanol mixture and maintained transfection efficiency after aerosolization using standard MDI container closure components [75]. These studies indicate that biological functionality of DNA is preserved upon contact with typical medicinal propellants. Proteins also seem to be compatible with propellants as shown, for example, for lysozyme in HFA 134a and HFA 227ea [76] and insulin in HFA 134a [77]. Recently, bacteriophages were successfully actuated from an MDI with a titer loss of less than 1 $\log_{10}$ [78]. In this study ΦKZ/D3 and KS4-M phages were aerosolized in the form of a reverse aqueous emulsion in HFA 134a. The results show that *Myoviridae* phages can tolerate the shear stresses associated with flash atomization in MDIs.

14.5 Summary and Outlook

Antibiotic-resistant bacterial infections could become a yet more severe threat to global health in coming decades, with the emergence of extensively drug-resistant tuberculosis as a prime example. It is questionable whether the development of small-molecule antibiotics can keep pace with the rate at which bacteria become resistant to them. Bacteriophages have many advantages that make them worthwhile candidates in the search for alternative antibiotics. Our understanding of phage biology and methods of genetic manipulation have advanced tremendously in recent years. Selection of suitable phages based on an understanding of their genomes or even design of useful phage components and use of vectors for targeted delivery for therapy or prophylaxis or both is within reach. Hoverer, many aspects of phage-based products, concerning areas of safety, efficacy, manufacturability, preferred delivery route, regulatory framework, and economics are still unclear. Given the potential utility of inhaled phages as a specific and very potent intervention against multi-drug-resistant pulmonary bacteria, including tuberculosis, it appears reasonable to intensify efforts to assess and develop this alternative.

Acknowledgements

We thank Nicholas Carrigy for comments on the manuscript and Zaritza Petrova for help with the plaque figures.

References

[1] Koskella, B. and Meaden, S. (2013) Understanding bacteriophage specificity in natural microbial communities. *Viruses*, **5**, 806–823.
[2] Hatfull, G.F. and Jacobs, W.R., Jr., (1994) Mycobacteriophages: Cornerstones of mycobacterial research, in *Tuberculosis: Pathogenesis, Protection and Control* (ed. B.R. Bloom), ASM, Washington, pp. 165–183.
[3] Jacobs-Sera, D., Marinelli, L.J., Bowman, C. *et al*. (2012) On the nature of mycobacteriophage diversity and host preference. *Virology*, **434**, 187–201.
[4] Hatfull, G.F. (2014) Molecular Genetics of Mycobacteriophages. *Microbiol. Spectrum*, **2**, 1–36.

[5] Hatfull, G.F. (2012) The secret lives of mycobacteriophages. *Adv. Virus Res.*, **82**, 179–288.
[6] Dedrick, R.M., Marinelli, L.J., Newton, G.L. *et al*. (2013) Functional requirements for bacteriophage growth: gene essentiality and expression in mycobacteriophage Giles. *Mol. Microbiol.*, **88**, 577–589.
[7] Hatfull, G.F. and Hendrix, R.W. (2011) Bacteriophages and their Genomes. *Curr. Opin. Virol.*, **1**, 298–303.
[8] Hatfull, G.F. (2010) Mycobacteriophages: genes and genomes. *Annu. Rev. Microbiol.*, **64**, 331–356.
[9] Pope, W.H., Ferreira, C.M., Jacobs-Sera, D. *et al.* (2011) Cluster K Mycobacteriophages: Insights into the Evolutionary Origins of Mycobacteriophage TM4. *PLoS One*, **6**, e26750.
[10] Fullner, K.J. and Hatfull, G.F. (1997) Mycobacteriophage L5 infection of Mycobacterium bovis BCG: implications for phage genetics in the slow-growing mycobacteria. *Mol. Microbiol.*, **26**, 755–766.
[11] Ford, M.E., Sarkis, G.J., Belanger, A.E. *et al*. (1998) Genome structure of mycobacteriophage D29: implications for phage evolution. *J. Mol. Biol.*, **279**, 143–164.
[12] Sampson, T., Broussard, G.W., Marinelli, L.J. *et al*. (2009) Mycobacteriophages BPs, Angel and Halo: comparative genomics reveals a novel class of ultra-small mobile genetic elements. *Microbiology*, **155**, 2962–2977.
[13] Peña, C.E., Stoner, J. and Hatfull, G.F. (1998) Mycobacteriophage D29 integrase-mediated recombination: specificity of mycobacteriophage integration. *Gene*, **225**, 143–151.
[14] Donnelly-Wu, M.K., Jacobs, W.R., Jr. and Hatfull, G.F. (1993) Superinfection immunity of mycobacteriophage L5: applications for genetic transformation of mycobacteria. *Mol. Microbiol.*, **7**, 407–417.
[15] Broussard, G.W. and Hatfull, G.F. (2013) Evolution of genetic switch complexity. *Bacteriophage*, **3**, e24186.
[16] Broussard, G.W., Oldfield, L.M., Villanueva, V.M. *et al.* (2013) Integration-dependent bacteriophage immunity provides insights into the evolution of genetic switches. *Mol. Cell*, **49**, 237–248.
[17] Bowman, B.U. (1969) Properties of mycobacteriophage DS6A. I. Immunogenicity in rabbits. *Proc. Soc. Exp. Biol. Med.*, **131**, 196–200.
[18] Bowman, B.U., Newman, H.A., Moritz, J.M and Koehler, R.M. (1973) Properties of mycobacteriophage DS6A. *II. Lipid composition. Am. Rev. Respir. Dis.*, **107**, 42–49.
[19] Hatfull, G.F., the Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science Program, the KwaZulu-Natal Research Institute for Tuberculosis and HIV Mycobacterial Genetics Course Students and the Phage Hunters Integrating Research and Education Program (2012) Complete genome sequences of 138 mycobacteriophages. *J. Virol.*, **86**, 2382–2384.
[20] Barsom, E.K. and Hatfull, G.F. (1996) Characterization of Mycobacterium smegmatis gene that confers resistance to phages L5 and D29 when overexpressed. *Mol. Microbiol.*, **21**, 159–170.
[21] Rubin, E.J., Akerley, B.J., Novik, V.N. *et al*. (1999) In vivo transposition of mariner-based elements in enteric bacteria and mycobacteria. *Proc. Natl. Acad. Sci. USA*, **96**, 1645–1650.
[22] Cole, S.T., Brosch, R., Parkhill, J. *et al*. (1998) Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature*, **393**, 537–544.
[23] Hendrix, R.W., Smith, M.C., Burns, R.N. *et al*. (1999) Evolutionary relationships among diverse bacteriophages and prophages: all the world's a phage. *Proc. Natl. Acad. Sci. USA*, **96**, 2192–2197.
[24] Bibb, L.A., Hancox, M.I. and Hatfull, G.F. (2005) Integration and excision by the large serine recombinase phiRv1 integrase. *Mol. Microbiol.*, **55**, 1896–1910.
[25] Bibb, L.A. and Hatfull, G.F. (2002) Integration and excision of the Mycobacterium tuberculosis prophage-like element, phiRv1. *Mol. Microbiol.*, **45**, 1515–1526.
[26] Brudey, K., Driscoll, J.R., Rigouts, L. *et al*. (2006) Mycobacterium tuberculosis complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC Microbiol.*, **6**:23.
[27] Fineran, P.C., Blower, T.R., Foulds, I.J. *et al*. (2009) The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. *Proc. Natl. Acad. Sci. USA*, **106**, 894–899.

[28] Hoskisson, P.A., Sumby, P. and Smith, M.C. (2015) The phage growth limitation system in Streptomyces coelicolor A(3)2 is a toxin/antitoxin system, comprising enzymes with DNA methyltransferase, protein kinase and ATPase activity. *Virology*, **477**, 100–109.
[29] Rado, T.A., Bates, J.H., Engel, H.W. *et al*. (1975) World Health Organization studies on bacteriophage typing of mycobacteria. Subdivision of the species Mycobacterium tuberculosis. *Am. Rev. Respir. Dis.*, **111**, 459–468.
[30] Wilson, S.M., al-Suwaidi, Z., McNerney, R. *et al*. (1997) Evaluation of a new rapid bacteriophage-based method for the drug susceptibility testing of Mycobacterium tuberculosis. *Nat. Med.*, **3**, 465–468.
[31] Muzaffar, R., Batool, S., Aziz, F. *et al.* (2002) Evaluation of the FASTPlaqueTB assay for direct detection of Mycobacterium tuberculosis in sputum specimens. *Int. J. Tuberc. Lung Dis.*, **6**, 635–640.
[32] McNerney, R. (2002) Phage tests for diagnosis and drug susceptibility testing. *Int. J. Tuberc. Lung Dis*., **6**, 1129–1130; author reply, 1130.
[33] Jacobs, W.R., Jr., Barletta, R.G., Udani, R. *et al*. (1993) Rapid assessment of drug susceptibilities of Mycobacterium tuberculosis by means of luciferase reporter phages. *Science*, **260**, 819–822.
[34] Piuri, M., Jacobs, W.R., Jr. and Hatfull, G.F. (2009) Fluoromycobacteriophages for rapid, specific, and sensitive antibiotic susceptibility testing of Mycobacterium tuberculosis. *PLoS One*, **4**, e4870.
[35] Jain, P., Hartman, T.E., Eisenberg, N. *et al*. (2012) phi(2)GFP10, a high-intensity fluorophage, enables detection and rapid drug susceptibility testing of Mycobacterium tuberculosis directly from sputum samples. *J. Clin. Microbiol.*, **50**, 1362–1369.
[36] Banaiee, N., Bobadilla-Del-Valle, M., Bardarov, S., Jr. *et al*. (2001) Luciferase reporter mycobacteriophages for detection, identification, and antibiotic susceptibility testing of Mycobacterium tuberculosis in Mexico. *J. Clin. Microbiol.*, **39**, 3883–3888.
[37] Banaiee, N., Bobadilla-del-Valle, M., Riska, P.F. *et al*. (2003) Rapid identification and susceptibility testing of Mycobacterium tuberculosis from MGIT cultures with luciferase reporter mycobacteriophages. *J. Med. Microbiol.*, **52**, 557–561.
[38] Summers, W.C. (1999) *Felix d'Herelle and the Origins of Molecular Biology*. Yale University Press, New Haven.
[39] Endersen, L., O'Mahony, J., Hill, S. *et al*. (2014) Phage therapy in the food industry. *Annu. Rev. Food Sci. Technol.*, **5**, 327–349.
[40] Hurley, J.C. (1992) Antibiotic-induced release of endotoxin: a reappraisal. *Clin. Infect. Dis.*, **15**, 840–854.
[41] Hatfull, G.F. (2014) Mycobacteriophages: windows into tuberculosis. *PLoS Pathog.*, **10**, e1003953.
[42] Sula, L., Sulova, J. and Stolcpartova, M. (1981) Therapy of experimental tuberculosis in guinea pigs with mycobacterial phages DS-6A, GR-21 T, My-327. *Czech. Med.*, **4**, 209–214.
[43] Hagens, S., Habel, A. and Blasi, U. (2006) Augmentation of the antimicrobial efficacy of antibiotics by filamentous phage. *Microb. Drug Resist.*, **12**, 164–168.
[44] Danelishvili, L., Young, L.S. and Bermudez, L.E (2006) In vivo efficacy of phage therapy for Mycobacterium avium infection as delivered by a nonvirulent mycobacterium. *Microb. Drug Resist.*, **12**, 1–6.
[45] Scholl, D., Cooley, M., Williams, S.R. *et al.* (2009) An engineered R-type pyocin is a highly specific and sensitive bactericidal agent for the food-borne pathogen Escherichia coli O157:H7. *Antimicrob. Agents Chemother.*, **53**, 3074–3080.
[46] Gebhart, D., Williams, S.R., Bishop-Lilly, K.A. *et al*. (2012) Novel high-molecular-weight, R-type bacteriocins of Clostridium difficile. *J. Bacteriol.*, **194**, 6240–6247.
[47] Loeffler, J.M., Nelson, D. and Fischetti, V.A. (2001) Rapid killing of Streptococcus pneumoniae with a bacteriophage cell wall hydrolase. *Science*, **294**, 2170–2172.
[48] Loeffler, J.M., Djurkovic, S. and Fischetti, V.A. (2003) Phage lytic enzyme Cpl-1 as a novel antimicrobial for pneumococcal bacteremia. *Infect. Immun.*, **71**, 6199–6204.
[49] Nelson, D., Loomis, L. and Fischetti, V.A. (2001) Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. *Proc. Natl. Acad. Sci. USA*, **98**, 4107–4112.

[50] Yoong, P., Schuch, R., Nelson, D. and Fischetti, V.A. (2006) PlyPH, a bacteriolytic enzyme with a broad pH range of activity and lytic action against Bacillus anthracis. *J. Bacteriol.*, **188**, 2711–2714.
[51] Gil, F., Catalao, M.J., Moniz-Pereira, J. *et al.* (2008) The lytic cassette of mycobacteriophage Ms6 encodes an enzyme with lipolytic activity. *Microbiology*, **154**, 1364–1371.
[52] Payne, K.M. and Hatfull, G.F. (2012) Mycobacteriophage endolysins: diverse and modular enzymes with multiple catalytic activities. *PLoS One*, **7**, e34052.
[53] Payne, K., Sun, Q., Sacchettini, J. and Hatfull, G.F. (2009) Mycobacteriophage Lysin B is a novel mycolylarabinogalactan esterase. *Mol. Microbiol.*, **73**, 367–381.
[54] Gil, F., Grzegorzewicz, A.E., Catalao, M.J. *et al*. (2010) Mycobacteriophage Ms6 LysB specifically targets the outer membrane of Mycobacterium smegmatis. *Microbiology*, **156**, 1497–1504.
[55] Jacobs, W.R., Jr., Tuckman, M. and Bloom, B.R. (1987) Introduction of foreign DNA into mycobacteria using a shuttle phasmid. *Nature*, **327**, 532–535.
[56] Marinelli, L.J., Hatfull, G.F. and Piuri, M. (2012) Recombineering: A powerful tool for modification of bacteriophage genomes. *Bacteriophage*, **2**, 5–14.
[57] Marinelli, L.J., Piuri, M., Swigonova, Z. *et al*. (2008) BRED: a simple and powerful tool for constructing mutant and recombinant bacteriophage genomes. *PLoS One*, **3**, e3957.
[58] Nicas, M., Nazaroff, W.W. and Hubbard, A. (2005) Toward understanding the risk of secondary airborne infection: emission of respirable pathogens. *J. Occup. Environ. Hyg.*, **2**, 143–154.
[59] McNerney, R. and Traore, H. (2005) Mycobacteriophage and their application to disease control. *J. Appl. Microbiol.*, **99**, 223–233.
[60] Guo, S. and Ao, Z. (2012) Phage in the diagnosis and treatment of tuberculosis. *Front. Biosci.*, **17**, 2691–2697.
[61] Emery, D.L. and Whittington, R.J. (2004) An evaluation of mycophage therapy, chemotherapy and vaccination for control of Mycobacterium avium subsp. paratuberculosis infection. *Vet. Microbiol.*, **104**, 143–155.
[62] Liu, K., Wen, Z., Li, N. *et al*. (2012) The impact of relative humidity and collection media on mycobacteriophage D29 aerosol. *Appl. Environ. Microbiol.*, **78**, 1466–1472.
[63] Hoe, S., Semler, D.D., Goudie, A.D. *et al*. (2013) Respirable bacteriophages for the treatment of bacterial lung infections. *J. Aerosol Med. Pulm. Drug Deliv.*, **26**, 317–335.
[64] Golshahi, L., Seed, K.D., Dennis, J.J. and Finlay, W.H. (2008) Toward modern inhalational bacteriophage therapy: nebulization of bacteriophages of Burkholderia cepacia complex. *J. Aerosol Med. Pulm. Drug Deliv.*, **21**, 351–360.
[65] Semler, D.D., Goudie, A.D., Finlay, W.H. and Dennis, J.J. (2014) Aerosol phage therapy efficacy in Burkholderia cepacia complex respiratory infections. *Antimicrob. Agents Chemother.*, **58**, 4005–4013.
[66] Puapermpoonsiri, U., Ford, S.J. and van der Walle, C.F. (2010) Stabilization of bacteriophage during freeze drying. *Int. J. Pharm.*, **389**, 168–175.
[67] Fortier, L.C. and Moineau, S. (2009) Phage production and maintenance of stocks, including expected stock lifetimes. *Methods Mol. Biol.*, **501**, 203–219.
[68] Engel, H.W., Smith, L. and Berwald, L.G. (1974) The preservation of mycobacteriophages by means of freeze drying. *Am. Rev. Respir. Dis.*, **109**, 561–566.
[69] Merabishvili, M., Vervaet, C., Pirnay, J.P. *et al*. (2013) Stability of Staphylococcus aureus phage ISP after freeze-drying (lyophilization). *PLoS One*, **8**, e68797.
[70] Golshahi, L., Lynch, K.H., Dennis, J.J. and Finlay, W.H. (2011) In vitro lung delivery of bacteriophages KS4-M and PhiKZ using dry powder inhalers for treatment of Burkholderia cepacia complex and Pseudomonas aeruginosa infections in cystic fibrosis. *J. Appl. Microbiol.*, **110**, 106–117.
[71] Matinkhoo, S., Lynch, K.H., Dennis, J.J. *et al*. (2011) Spray-dried respirable powders containing bacteriophages for the treatment of pulmonary infections. *J. Pharm. Sci.*, **100**, 5197–5205.
[72] Vandenheuvel, D., Singh, A., Vandersteegen, K. *et al*. (2013) Feasibility of spray drying bacteriophages into respirable powders to combat pulmonary bacterial infections. *Eur. J. Pharm. Biopharm.*, **84**, 578–582.
[73] Vandenheuvel, D., Meeus, J., Lavigne, R. and Van den Mooter, G. (2014) Instability of bacteriophages in spray-dried trehalose powders is caused by crystallization of the matrix. *Int. J. Pharm.*, **472**, 202–205.

[74] Conti, D.S., Bharatwaj, B., Brewer, D. and da Rocha, S.R. (2012) Propellant-based inhalers for the non-invasive delivery of genes via oral inhalation. *J. Control. Release*, **157**, 406–417.
[75] Bains, B.K., Birchall, J.C., Toon, R. and Taylor, G. (2010) In vitro reporter gene transfection via plasmid DNA delivered by metered dose inhaler. *J. Pharm. Sci.*, **99**, 3089–3099.
[76] Quinn, E.A., Forbes, R.T., Williams, A.C. *et al*. (1999) Protein conformational stability in the hydrofluoroalkane propellants tetrafluoroethane and heptafluoropropane analysed by Fourier transform Raman spectroscopy. *Int. J. Pharm.*, **186**, 31–41.
[77] Nyambura, B.K., Kellaway, I.W., and Taylor, K.M. (2009) Insulin nanoparticles: stability and aerosolization from pressurized metered dose inhalers. *Int. J. Pharm.*, **375**, 114–122.
[78] Hoe, S., Boraey, M.A., Ivey, J.W. *et al*. (2014) Manufacturing and device options for the delivery of biotherapeutics. *J. Aerosol Med. Pulm. Drug Deliv.*, **27**, 315–328.

15

RNA Nanoparticles as Potential Vaccines

Robert DeLong

Department of Anatomy and Physiology, College of Veterinary Medicine, Kansas State University, 228 Coles Hall, Manhattan, KS, 66506-5802, USA

15.1 Introduction

Since the early 1800s when preparations of cowpox were used to vaccinate people against smallpox, vaccines have evolved in parallel as our understanding of molecular and cellular biology has advanced. In the last decade, the field of genomics has quite literally exploded and along with that DNA-based vaccines have also progressed and moved into clinical trials [1]. RNA has traditionally been the "black box" of molecular biology however, and our understanding of the complex structure–function of RNA molecules in cells and tissues is only recently becoming more complete. In perhaps the broadest definition of a vaccine listed within the Merriam-Webster dictionary "medical: a substance that is usually injected into a person or animal to protect against a particular disease". There is thus no reason RNA cannot be considered as a vaccine. In this treatise we discuss the effects of nanoparticles on the structure, function and delivery of RNA as relevant to the progression of RNA-based vaccines.

15.2 Nanoparticles

Today nanoparticles have been prepared or synthesized chemically from almost every element in Mendeleev's periodic chart. The early biomedical nanotechnology literature was dominated by gold nanoparticles because of the long held belief that gold is

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

biologically inert. It is clear that unfunctionalized gold nanoparticles do enter cells [2]; however, ironically gold is not naturally present in cells and tissues nor does it naturally interact with RNA and thus as we reviewed in 2010, gold nanoparticles must be surface-functionalized in order to bind RNA for RNA delivery [3]. In cells and tissues, RNA–protein interactions are well-known to be stabilized by manganese [4], and thus our group and two others were the first to report manganese-based nanoparticles for intracellular association and delivery [5–7]. The manganese(II) oxide nanorods we used had a negative surface charge or zeta potential so to induce RNA to bind, we first had to coat their surface with poly(amidoamine) (PAMAM) dendrimer [7] and the other groups similarly used other cationic delivery-enhancing polymers for the same purpose [5, 6]. More recently, using Raman spectroscopy characterization, we have observed a weak interaction between zinc oxide (ZnO) nanoparticle and ATP, one of the simplest model RNA molecules [8]. Thus we are currently exploring alternative nucleic acid or nanoparticle chemistries to improve RNA interaction and delivery.

15.3 RNA Nanoparticle Vaccines

In theory, therefore, delivery of an RNA molecule into an antigen presentation cell (APC) or another cell of the immune system by a nanoparticle or conjugate could be considered a type of vaccine approach. The typical understanding of a vaccine is that an effective biological response from the injected substance triggers an antibody response. However, a cell-mediated response is also desirable in many cases. Historically, the identification of various sequence-specific restriction endonucleases permitted molecular cloning and the insertion and expression of antigen-encoding sequences in cells after delivery of the DNA vector. As mentioned previously, with the ramp up of molecular cloning came the explosion in the field of genomics which was mostly DNA-centric and hence DNA vaccines came out in front of RNA vaccines. Today, however, *in vitro* RNA expression and purification technology has advanced considerably. This has enabled the production of RNA at a large enough scale to support bench characterization, as well as cell and animal studies on a single batch necessary to support pre-clinical and clinical testing. Indeed, RNA molecules containing an appropriate 5' untranslated region (5'-UTR), 3' untranslated region and poly A tail can be engineered such that the RNA molecule itself can be the agent that gets delivered and expressed in cells. This has several important advantages. Perhaps the most important of these is that if stability of the RNA and delivery into an appropriate cell can be ensured, the other main obstacles associated with DNA vaccines can be completely avoided. Thus, for example, nuclear delivery and entry through the nuclear membrane is completely unnecessary. Further, replication and transcription steps can be by-passed altogether and are no longer required, since an appropriate RNA vector will be translatable in the cytosol where it has been delivered. This greatly simplifies the delivery process. RNA vaccines also have important safety advantages over DNA vaccines. In fact this safety advantage may be the single most attractive feature or selling point for RNA vaccines. Here issues that have hindered DNA vaccines, such as integration, auto-immunity, low immunogenicity and others, are completely avoided as well. In fact, for TB, one of the earliest studies demonstrated the power of RNA vaccination [9]. However, it should be stressed that this seminal work very early on identified the two greatest limiting factors for RNA

vaccination. The first issue being the critical need for stabilization of the RNA given its susceptibility to nuclease or RNase degradation. The second issue being delivery of the RNA into an appropriate APC. Towards that end, nanotechnology, and the stabilization and delivery of RNA by nanoparticles, may be the "saving grace" for RNA vaccination, as discussed intensively below.

15.4 Progression of Nanomedicines into the Clinic

To date only six nanomedicines have been Food and Drug Administration (FDA)-approved for use in humans; two antibody–-drug conjugates, three liposomal–drug conjugates and one protein–drug carrier [10]. Of note, there are no nanoparticle-based RNA therapeutics currently available, despite some early success in the clinic and translation across multiple animal species [11, 12]. Although the initial pharmacokinetics and toxicology profiles seem promising, the main problem for these small interfering RNA (siRNA) or microRNA (miRNA) nanoparticles, which is similar to that for gene therapy, is that the inhibition of one gene or introduction of one gene may not be enough to sustain a therapeutic effect. Of course this is not the case for an RNA vaccine since the expression of the antigen and subsequent immunological effect and the memory created therefrom may be enough to be protective, and possibly curative. In recent years, therefore, several RNA-based vaccines have progressed into clinical trials as represented in Table 15.1.

As shown in Table 15.1 although the clinical experience with RNA vaccines is limited, the results thus far are promising, including but not limited to: good safety profiles, antigen expression, reduced tumor burden, CD4 and CD8 and interferon-gamma (IFN-γ) response, induction of neutralizing antibodies and others [13–16]. A larger body of pre-clinical work is in support of these studies and is similarly demonstrating promising results [17–21]. Topical or lung delivery and electroporation of the nanoparticles seem to be the methods of choice, but may require specialized equipment and devices [22–24]. However, these issues are outside the scope of the current review where our focus is on stability, cellular delivery and the characterization of the vaccine components (RNA, adjuvant and nanoparticle) as discussed in the next sections.

15.5 The Stability Problem

Nucleic acids are susceptible to nuclease degradation and thus one important aspect for their clinical success is that the nanoparticle or nanoconjugate used for delivery must be able to impart resistance to DNase or RNase digestion. For other forms of nucleic acid therapies such as antisense, siRNA or miRNA delivery, this is less of an issue, because these oligonucleotides can be synthesized with stability-enhancing chemical modifications such as in the phosphate backbone or sugar, which greatly improves nuclease-resistance and prolongs pharmacologic activity. Unfortunately this is not possible for DNA or RNA vaccines since these molecules are much longer and generally cannot be chemically synthesized as oligonucleotides. Instead the sequences must be biologically derived and hence stabilization must come from the nanoparticle or conjugate to which the DNA or RNA is attached or bound. Unfortunately again the great limitations for RNA vaccines therefore is

Table 15.1 *Some early clinical results of RNA vaccines in humans*

RNA type	Disease	Adjuvant	Delivery	Biological response	Reference
cDNA total mRNA pool	Metastatic melanoma	GM-CSF[a]	Direct intradermal application	Phase I safety criteria	[13]
mRNA encoding MUC1, CEA, Her-2/neu, telomerase, survivin and MAGE-A1	Stage IV renal carcinoma	GM-CSF	Direct intradermal application	Response Evaluation Criteria In Solid Tumors criteria (RECIST), improved survival, CD4 and CD8 response	[14]
CMV gB or a pp65/IE1 fusion protein	Cytomegalovirus (CMV)	none	Intradermal or intramuscular injection of alphavirus replicon particle	IFN-γ ELISPOT to CMV antigens, neutralizing antibodies, CD4+ and CD8+ TCR	[15]
CMV pp65 messenger RNA-loaded autologous monocyte-derived dendritic cells	Human cytomegalovirus (CMV)	none	Leukapheresis procedure followed by intradermal administration	CMV-specific T cell response	[16]

[a] Granulocyte-macrophage colony-stimulating factor.

that RNA is much less stable than DNA and much more susceptible to the myriad of nucleases present in biological fluids and tissues which can rapidly destroy it. This makes the delivery approach and whether it can stabilize the RNA absolutely critical [25]. For viral delivery, at least while outside the cell and possibly within the cell as RNA:protein complexes, the RNA is likely protected from such degradation, thus the challenge for any nanoparticle-based approach is to mimic this and similarly stabilize the RNA.

One common approach to solving this stability problem emerging in the literature is layer-by-layer (LBL or l-b-l) assembly strategies, whereby the nucleic acid can be protected under a coating of a stability-enhancing material. One means of doing this we have explored is to bring the nucleic acid in conjunction with the core nanoparticle by utilizing a protein such as protamine or a polymer such as PAMAM dendrimer [7, 26, 27]. Although these surface structures have been difficult for us to image with high resolution [28], our working hypothesis is that the dendrimeric structure provides a cage that protects the nucleic acid from nuclease degradation, as illustrated in Figure 15.1.

As illustrated schematically in Figure 15.1, certainly based on our data, complexation of RNA to dendrimer improves the RNA stability and lessens degradation from RNase A [29]. Furthermore, recently we have shown that dendrimer structures modified with poly(ethylene glycol) (PEG-dendrimer) when complexed to RNA can protect against degradation from RNase, serum nucleases and tissue extracts, moderately improved by binding to a core gold nanoparticle [28].

Layer-by-layer assembly was perhaps most elegantly described more recently by Paula Hammond's group at MIT where they created DNA microsponges which exhibit excellent cell uptake and *in vivo* stability [30]. Similarly, Sokolova and Epple have prepared calcium

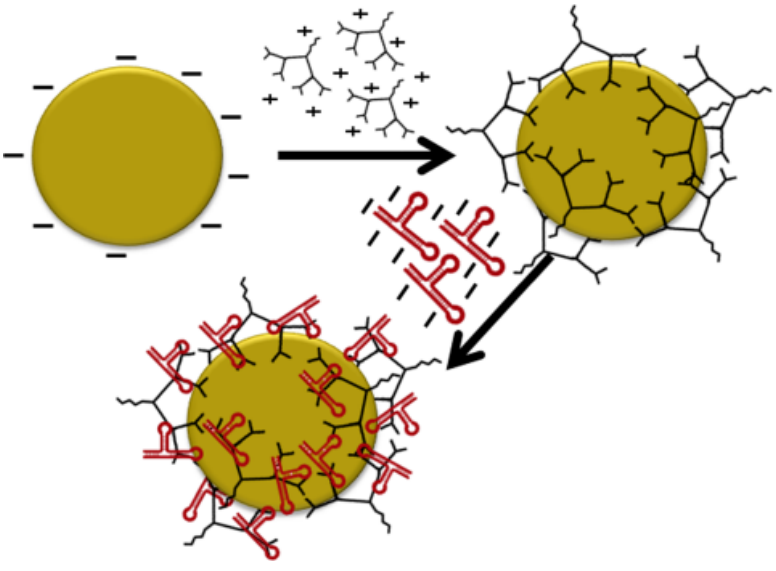


Figure 15.1 *Formation of a nucleic acid (RNA is shown) conjugate where a branched dendrimer structure binds it to the surface of the nanoparticle and protects it from nuclease degradation. (See insert for color representation of the figure.)*

phosphate nanoshells that can sequester DNA and RNA within the interior environment of the nanoparticle which then exhibit robust specific immunological activity [31–33]. Lipid-protamine-RNA formulations after lyophilization and resuspension have also shown good RNA stabilization and activity against Hepatitis C after prolonged storage [34]. There are several other alternative approaches to stabilizing RNA into nanospheres and other structures that may also be amenable for RNA vaccination [35–37].

15.6 The Delivery Problem

For a nanoparticle to deliver RNA into cells it must first bind RNA. As discussed above, some nanoparticles may be able to bind RNA directly, whereas others must be surface-coated or functionalized to be able to attach RNA. Alternatively, LBL assembly or calcium phosphate shell-type approaches may be able to internalize RNA within the interior of the nanoparticle. Clearly an RNA expression vector delivered by either a viral or non-viral particle is able to by-pass nuclear delivery and any replication or transcription steps needed to express the antigen or immunity production. However, perhaps even more important than stabilizing the RNA is that cellular entry must be gained and this by its very nature means getting across the cell or plasma membrane.

Chithrani elegantly and conclusively demonstrated gold nanoparticles (GNPs) are able to enter cells [2] and there has since been a great deal of work done on GNPs accordingly, which is too numerous to cite. More recently, coated iron oxide nanoparticles have been used by Bob Langer's group at MIT for RNA delivery, and these have desirable magnetic properties which can be used to direct them to desirable tissues [38]. In addition to our work on PAMAM dendrimer- or PEG-dendrimer- coated gold or manganese(II) oxide nanoparticles for DNA and RNA delivery [7, 28], others have recently coated silica dioxide nanoparticles with poly(ethyleneimine) (PEI) to achieve effective gene and siRNA delivery [39]. To demonstrate that the RNA is functionally active once delivered into the target cell we use a functional RNA-delivery assay [26, 28]. In this cell-culture system amenable for RNA nanotechnology delivery, an RNA switch is engineered into either HeLa or A375 cells and marker-gene expression, luciferase, is under the control of a splice-switching oligomer which, when delivered, corrects the aberrant splicing turning on luciferase and hence the cells' light production [26, 28]. In this system protamine-coated or PEG-PAMAM-coated gold nanoparticles have shown activity [26, 28]. More recently, protamine–mRNA complex when combined with adjuvants imiquimod and GM-CSF was used for RNA vaccination and tested in the clinic against prostate cancer [40]. As a phase I/II trial, in this study safety was the primary emphasis and no adverse effects were reported. However, the study raised the question of toll-like receptor (TLR) agonists as a means of achieving robust biological responses [40], which we address in the next section.

15.7 RNA as Targeting Agent or Adjuvant?

As summarized previously in Table 15.1, clinical end-points in most RNA vaccine studies to date have determined T cell responses, antibody, interferon or cytokine profiles. There is a huge literature accumulating now that for effective adjuventation and to increase the

biological response, TLR agonists and/or targeting agents will be required as part of the vaccine formulation to trigger robust activity. Although poly(inosinic:cytidylic acid) (poly I:C) was initially touted as an immune cell-targeting agent in combination with nanoparticles [41, 42], what is very clear is that this double-stranded RNA (dsRNA) molecule is very active immunologically and can induce beneficial interferon response and cellular recruitment that can augment antiviral and anticancer activity [43–46]. Poly I:C has been tested extensively in the clinic and although it does not trigger a specific antibody-based response per se, it is being considered as more than just an adjuvant and as a nanoconjugate may be considered as a potential RNA-based biological therapy. For example, very recently an iron oxide poly I:C nanocarrier was developed demonstrating enhanced immunological stimulation and lymph-node targeting [47].

There is more than two decades' worth of clinical experience with poly I:C in humans. Although much of this early work focused on cancer, in some cases relevant biological or immunological end-points were examined, and these are summarized briefly in Table 15.2.

As reflected in Table 15.2, poly I:C has demonstrated clinically relevant results against cancer, viral infection and skin lesions. More generally, given the immuno-activity of poly I:C, there has recently been a tremendous upsurge in interest for particle-based delivery of poly I:C [54]. Porcine models are often used as a predecessor to clinical trials in immunology where we and many other groups first perform stability and activity studies in mouse and guinea pigs prior to pivotal pre-clinical trials in the pig [26, 27]. There is a large body of data in support of poly I:C in lower rodent species [53, 55, 56], but more recently in porcine models poly I:C has been shown to augment the T-helper and cytokine response against reproductive and respiratory virus infection [57, 58]. As above, actions for poly I:C seem particularly appropriate for skin or intradermal delivery, but given its ability to prime

Table 15.2 *Early clinical studies on poly I:C dsRNA*

Study	Disease	Delivery	Biological response	Reference
Clinical trial	Herpes zoster in children with cancer	Topical poly I:C	Decreased median days of lesions	[48]
Phase I-II	Leukemia (solid tumors)	Poly ICLC[a] injection	Induced significant levels of serum interferon	[49]
Phase IB trial	Variety of cancers	Poly ICLC + IL-2[b] injection	Increase NK[c] activity, moderate toxicity	[50]
Pilot clinical trial	Metastatic cancer	Type I DCs activated with poly I:C + prepared tumor lysates	Increased NK activity; increased lymph targeting	[51]
Phase I trial	Cancer	Vaccine containing poly I:C	Increased duration of stable disease; increased immune response	[52]
Clinical trial	Laser-induced skin wounds	Topical poly I:C	Improved wound healing time and quality	[53]

[a] Interlab Cell Line Collection.
[b] Interleukin-2.
[c] Natural killer.

T-helper cells and dendritic cell activity [59–61] its activity against TB, and more broadly for applications in lung delivery [62], needs to be further investigated.

In terms of nanoparticle targeting with RNA and adjuventation, in addition to poly I:C [41, 42], there is a large emerging literature in support of aptamers as potential cell- and tissue-targeting agents [63–69]. Thus RNA aptamers may also be useful for targeting RNA vaccine, with poly I:C or other active RNA nanoparticles to cells or tissues such as the lymph node [70], lung [22] or other desirable immunogenic sites. More recently, reports of aptamers augmenting protection against experimental TB in mice and monkeys [71] also suggest promise for aptamer-based targeting and therapy. Longer-term it seems likely therefore to combine an RNA-targeting agent with immunogenic agent to create a dual- or even tri-functional nanoparticle containing RNA-targeting agent, vaccine or immuno-active and chemotherapeutic components, which is the subject of our current research program.

15.8 Challenges for RNA Nanoparticle Vaccine Characterization

To progress into clinical trials it is imperative to be able to demonstrate control over the nanoparticle, RNA and other chemical components of the vaccine formulation and any processes used in their manufacture. Towards that end our patents in DNA vaccine particle delivery and characterization are informative [72–84]. Ideally, a single production batch of nanoparticles would be suitable for all pre-clinical and clinical studies. However, this capability is not yet generally available and is the subject of an intense amount of research and development world-wide. In this case, it is expected that homogeneity in the size, shape and surface charge and the elemental composition of the nanoparticle will be needed, since these factors are well-known to influence pharmacokinetics and biodistribution [85], and seem likely to impact biomolecular interaction [86]. Control over the payload or amount of RNA per mass of nanoparticle will also be necessary. We have used several characterization methods to achieve this for DNA, utilizing either UV spectroscopy or high-performance liquid chromatography (HPLC) with UV detection [26, 27], and have more recently adapted these and gel electrophoresis methods similarly for RNA [7, 28]. It will also be necessary to have cell culture and animal models qualified in order to demonstrate biochemical, biological and immunological activity. Structural or functional assays for RNA in particular are more challenging than are those for DNA or protein, as RNA is generally more flexible and easily denatured or degraded such that crystal structures are generally difficult to obtain particularly for larger RNA molecules. In the case of an antisense or siRNA oligonucleotide, activity can be measured by loss in the expression of the protein by western, enzyme-linked immunosorbent assay (ELISA) or biochemical activity assay, or alternatively at the RNA level by reverse transcription polymerase chain reaction (RT-PCR) for these and splice-switching or splice-shifting oligomers [26, 87, 88]. However this is generally not the case for an RNA vaccine where here our approach for DNA vaccines is amenable whereby the vector is delivered into cultured cells and antigen expression measured [26, 27], or into a co-culture system where efficacy of antigen presentation is read out by cytokine secretion [87, 89]. Mouse or guinea pig studies in parallel where antigen and cytokine parameters are obtained will then allow *in vitro*/*in vivo* corollary [26, 27]. Finally, prior to pivotal proof-of-principle pre-clinical studies, it is advisable to characterize the immunotoxicology of the RNA nanoparticle vaccine. Here, in addition to endotoxin assay, characterization of red

or white blood cell recruitment and γ-interferon induction in the rabbit knee, mouse intraperitoneal environment and *ex vivo* splenocyte assay is advised [90].

15.9 On the Horizon

In conclusion, the prospect for RNA vaccine nanoparticles in the near future seems quite promising. In addition to the RNA expression vector, multi-functional RNA nanoparticles may be created which contain TLR agonist RNA molecules and targeting molecules or aptamers. Combination of the nanoparticle and poly I:C may potentiate antigen presentation [89] and is potentially amenable to TB and possibly even DNA vaccination. For example, poly I:C has been shown to potentiate a CD40-based DNA vaccine against melanoma [91]. Although the field is dominated by iron oxide and gold nanoparticles, our studies and others have more recently focused on zinc and manganese(II) oxide nanoparticles [5–8, 28, 85]. Cobalt nanoparticles are also on the horizon, as these possess desirable magnetic properties and recent biological transmission electron microscopy experiments have shown that these enter cells and have a negligible dissolution rate in biological media [92]. More recently we have described gold-coated cobalt nanoparticles [93] and such composite materials, for example combining the desirable properties of one type of nanoparticle with another, or even more than one, are gaining in popularity. Thus it has been shown that doping zinc oxide with cobalt can increase the antibacterial properties and hence lower the minimum inhibitory concentration [94]. This suggests that it may be possible to design and test RNA vaccine nanoparticles which possess both an immunogenic and therapeutic component and that the underlying nanoparticle may contribute or synergize with the RNA. A similar approach has recently been championed by Peixuan Guo's group at the University of Kentucky, whereby the nanoparticle is constructed entirely of RNA, forming a rotary 3-D structure and a pore through which DNA, or RNA, is able to enter the cell [95]. Recently this group attenuated 5′-C-phosphate-G-3′ (CpG) to the arms and changed the shapes of these RNA structures from triangles to polygons and showed immunomodulation of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) [96]. In brief summary therefore it seems likely that RNA-based vaccines will be realized, as nanotechnology matures and becomes able to stabilize and deliver RNA such that it retains its expression and functionally activates the immune system [97, 98]. This, and the ability to combine nanoparticles with RNA-targeting molecules such as aptamers [99] or those capable of targeting specific cells within the immune system [100], raises sincere hopes that diseases which continue to destroy human health such as tuberculosis, hepatitis and cancer may become much more manageable as a result [101].

References

[1] Kutzler, M. and Weiner, D. (2008) DNA vaccines: ready for prime time? *Nat. Rev. Genet.*, **9** (10), 776–788.

[2] Chithrani, B., Ghazani, A. and Chan, W.C. (2006) Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. *Nano Lett.*, **6** (4), 662–668.

[3] DeLong, D., Reynolds, C., Malcolm, Y. *et al*. (2010) Functionalized gold nanoparticles for the binding, stabilization, and delivery of therapeutic DNA, RNA, and other biological macromolecules. *Nanotechnol. Sci. Appl.*, **3**, 53–63.

[4] Nauwelaerts, K., Fisher, M., Froeyen, M. *et al*. (2007) Structural characterization and biological evaluation of small interfering RNAs containing cyclohexenyl nucleosides. *J. Am. Chem. Soc.*, **129** (30), 9340–9348.
[5] Xing, R., Liu, G., Quan, Q. *et al*. (2011) Functional MnO nanoclusters for efficient siRNA delivery. *Chem. Commun. (Cambridge)*, **47** (44), 12152–12154. doi: 10.1039/c1cc15408g. Epub 2011 Oct 12.
[6] Bae, K., Lee, K., Kim, C. and Park, T.G. (2011) Surface functionalized hollow manganese oxide nanoparticles for cancer targeted siRNA delivery and magnetic resonance imaging. *Biomaterials*, **32** (1), 176–184.
[7] Parker-Esquivel, B., Flores, K., Louiselle, D. *et al*. (2012) Association of poly I:C RNA and plasmid DNA onto MnO nanorods mediated by PAMAM. *Langmuir*, **28** (8), 3860–3870.
[8] Bhaumik, A., Shearin, A., DeLong, R. *et al*. (2014) Probing the interaction at the nano-bio interface using raman spectroscopy: ZnO nanoparticles and adenosine triphosphate biomolecules. *J. Phys. Chem. C. Nanomater. Interfaces*, **118** (32), 18631–18639.
[9] Youmans, A. and Youmans, G. (1969) Factors affecting immunogenic activity of mycobacterial ribosomal and ribonucleic acid preparations. *J. Bacteriol.*, **99** (1), 42–50.
[10] Dawidczyk, C., Kim, C., Park, J. *et al*. (2014) State-of-the-art in design rules for drug delivery platforms: lessons learned from FDA-approved nanomedicines. *J. Control. Release*, **187**, 133–144.
[11] Zuckerman, J., Gritli, I., Tolcher, A. *et al.* (2014) Correlating animal and human phase Ia/Ib clinical data with CALAA-01, a targeted, polymer-based nanoparticle containing siRNA. *Proc. Natl. Acad. Sci. USA*, **111** (31), 11449–11454.
[12] Davis, M., Zuckerman, J., Choi, C. *et al*. (2010) Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*, **464** (7291), 1067–1070.
[13] Weide, B., Carralot, J.P., Reese, A. *et al*. (2008) Results of the first phase I/II clinical vaccination trial with direct injection of mRNA. *J. Immunother.*, **31** (2), 180–188.
[14] Rittig, S.M., Haentschel, M., Weimer, K.J. *et al*. (2011) Intradermal vaccinations with RNA coding for TAA generate $CD8^+$ and $CD4^+$ immune responses and induce clinical benefit in vaccinated patients. *Mol. Ther.*, **19** (5), 990–999.
[15] Bernstein, D.I., Reap, E.A., Katen, K. *et al*. (2009) Randomized, double-blind, phase 1 trial of an alphavirus replicon vaccine for cytomegalovirus in CMV seronegative adult volunteers. *Vaccine*, **28** (2), 484–493.
[16] Van Craenenbroeck, A., Smits, E., Anguille, S. *et al*. (2015) Induction of cytomegalovirus-specific T cell responses in healthy volunteers and allogeneic stem cell recipients using vaccination with messenger RNA-transfected dendritic cells. *Transplantation*, **99** (1), 120–127.
[17] Ljungberg, K. and Liljeström, P. (2015) Self-replicating alphavirus RNA vaccines. *Expert Rev. Vaccines*, **14** (2), 177–194.
[18] Pollard, C., Rejman, J., De Haes, W. *et al*. (2013) Type I IFN counteracts the induction of antigen-specific immune responses by lipid-based delivery of mRNA vaccines. *Mol. Ther.*, **21** (1), 251-259.
[19] Geall, A., Verma, A., Otten, G. *et al*. (2012) Nonviral delivery of self-amplifying RNA vaccines. *Proc. Natl. Acad. Sci. USA*, **109** (36), 14604–14609.
[20] Couty, J., Crain, A., Gerbaud, S. *et al*. (2008) Delivery of mengovirus-derived RNA replicons into tumoural liver enhances the anti-tumour efficacy of a peripheral peptide-based vaccine. *Cancer Immunol. Immunother*., **57** (8), 1161–1171.
[21] Kramps, K. and Probst, J. (2013) Messenger RNA-based vaccines: progress, challenges, applications. *Wiley Interdisciplinary Rev.: RNA*, **4** (6), 737–749. doi: 10.1002/wrna.1189. Epub 2013 Jul 25.
[22] Thorley, A., Ruenraroengsak, P., Potter, T. and Tetley, T. (2014) Critical determinants of uptake and translocation of nanoparticles by the human pulmonary alveolar epithelium. *ACS Nano*, **8** (11), 11778–11789. doi: 10.1021/nn505399e. Epub 2014 Nov 4. PMID: 25360809.
[23] Zheng, D., Giljohann, D., Chen, D. *et al*. (2012) Topical delivery of siRNA-based spherical nucleic acid nanoparticle conjugates for gene regulation. *Proc. Natl. Acad. Sci. USA*, **109** (30), 11975–11980.
[24] Broderick, K. and Humeau, L. (2015) Electroporation-enhanced delivery of nucleic acid vaccines. *Expert Rev. Vaccines*, **14** (2), 195–204.

[25] Liu, M. (2010) Immunologic basis of vaccine vectors. *Immunity*, **33** (4), 504–515.
[26] DeLong, R., Akhtar, U., Sallee, M. *et al.* (2009) Characterization and performance of nucleic acid nanoparticles combined with protamine and gold. *Biomaterials*, **30** (32), 6451–6459.
[27] Knowle, R., Werner, A. and DeLong, R. (2006) R4 peptide-pDNA nanoparticle coated HepB vaccine microparticles: sedimentation, partitioning, and spray freeze dry bioprocesses. *J. Nanosci. Nanotechnol.*, **6** (9–10), 2783–2789.
[28] Reyes-Reveles, J., Sedaghat-Herati, R., Gilley, D. *et al*. (2013) mPEG-PAMAM-G4 nucleic acid nanocomplexes: enhanced stability, RNase protection, and activity of splice switching oligomer and poly I:C RNA. *Biomacromolecules*, **14** (11), 4108–4115.
[29] Kang, H., DeLong, R., Fisher, M.H. and Juliano, R.I. (2005) Tat-conjugated PAMAM dendrimers as delivery agents for antisense and siRNA oligonucleotides. *Pharm. Res.*, **22** (12), 2099–2106.
[30] Roh, Y.H., Lee, J.B., Shopsowitz, K.E. *et al*. (2014) Layer-by-Layer Assembled Antisense DNA Microsponge Particles for Efficient Delivery of Cancer Therapeutics. *ACS Nano*, **8** (10), 9767–9780.
[31] Sokolova, V., Knuschke, T., Kovtun, A. *et al.* (2010) The use of calcium phosphate nanoparticles encapsulating Toll-like receptor ligands and the antigen hemagglutinin to induce dendritic cell maturation and T cell activation. *Biomaterials*, **31** (21), 5627–5633.
[32] Knuschke, T., Bayer, W., Rotan, O. *et al*. (2014) Prophylactic and therapeutic vaccination with a nanoparticle-based peptide vaccine induces efficient protective immunity during acute and chronic retroviral infection. *Nanomedicine*, **10** (8), 1787–1798.
[33] Temchura, V.V., Kozlova, D., Sokolova, V. *et al*. (2014) Targeting and activation of antigen-specific B-cells by calcium phosphate nanoparticles loaded with protein antigen. *Biomaterials*, **35** (23), 6098–6105.
[34] Kundu, A.K., Chandra, P.K., Hazari, S. *et al*. (2011) Stability of lyophilized siRNA nanosome formulations. *Int. J. Pharm.*, **423** (2), 525–534.
[35] Asia, T., Tsuzuku, T., Takahashi, S. *et al.* (2014) Cell-penetrating peptide-conjugated lipid nanoparticles for siRNA delivery. *Biochem. Biophys. Res. Commun.*, **444** (4), 599–604.
[36] Shimanovich, U., Volkov, V., Eliaz, D. *et al*. (2011) Stabilizing RNA by the sonochemical formation of RNA nanospheres. *Small*, **7** (8), 1068–1074.
[37] Johnson, B., Melde, B., Dinderman, M. and Lin, B. (2012) Stabilization of RNA through absorption by functionalized mesoporous silicate nanospheres. *PLoS One*, **7** (11), e50356.
[38] Jiang, S., Eltoukhy, A.A., Love, K.T. and Langer, D.G. (2013) Lipidoid-coated iron oxide nanoparticles for efficient DNA and siRNA delivery. *Nano Lett.*, **13** (3), 1059–1064.
[39] Xia, T., Kovochich, M., Liong, M. *et al*. (2009) Polyethyleneimine coating enhances the cellular uptake of mesoporous silica nanoparticles and allows safe delivery of siRNA and DNA constructs. *ACS Nano*, **3** (10), 3273–3286.
[40] Feyerabend, S., Stevanovic, S., Gouttefangeas, C. *et al*. (2009) Novel multi-peptide vaccination in Hla-A2+ hormone sensitive patients with biochemical relapse of prostate cancer. *Prostate*, **69** (9), 917–927.
[41] Shukoor, M.I., Natalio, F., Ksenofonto, V. *et al*. (2007) Double-stranded RNA polyinosinic-polycytidylic acid immobilized onto gamma-Fe_2O_3 nanoparticles by using a multifunctional polymeric linker. *Small*, **3** (8), 1374–1378.
[42] Ruyra, A., Cano-Sarabia, M., García-Valtanen, P. *et al.* (2014) Targeting and stimulation of the zebrafish (*Danio rerio*) innate immune system with LPS/dsRNA-loaded nanoliposomes. *Vaccine*, **32** (31), 3955–3962.
[43] Schaffert, D., Kiss, M., Rödl, W. *et al*. (2011) Poly(I:C)-mediated tumor growth suppression in EGF-receptor overexpressing tumors using EGF-polyethylene glycol-linear polyethylenimine as carrier. *Pharm. Res.*, **28** (4), 731–741.
[44] Corr, M., Boyle, D.L., Ronacher, L. *et al*. (2008) Synergistic benefit in inflammatory arthritis by targeting I kappaB kinase epsilon and interferon beta. *Ann. Rheum. Dis.*, **68** (2), 257–263.
[45] Shakhar, G., Abudarham, N., Melamed, R. *et al.* (2007) Amelioration of operation-induced suppression of marginating pulmonary NK activity using poly IC: a potential approach to reduce postoperative metastasis. *Ann. Surg. Oncol.*, **14** (2), 841–852.
[46] Devendra, D. and Eisenbarth, G.S. (2004) Interferon alpha – a potential link in the pathogenesis of viral-induced type 1 diabetes and autoimmunity. *Clin. Immunol.*, **111** (3), 225–233.

[47] Cobaleda-Siles, M., Henriksen-Lacey, M., Ruiz de Angulo, A. *et al*. (2014) An iron oxide nanocarrier for dsRNA to target lymph nodes and strongly activate cells of the immune system. *Small*, **10** (24), 5054– 5067.
[48] Feldman, S., Hughes, W.T., Darlington, R.W. and Kim, H.K. (1975) Evaluation of Topical Polyinosinic Acid-Polycytidylic Acid in Treatment of Localized Herpes Zoster in Children with Cancer: A Randomized, Double-Blind Controlled Study. *Antimicrob. Agents Chemother.*, **8** (3), 289–294.
[49] Levine, A.S. and Levy, H.B. (1978) Phase I-II Trials of Poly IC Stabilized with Poly-L-Lysine. *Cancer Treat. Rep.*, **62** (11), 1907–1912.
[50] Ewel, C.H., Urba, W.J., Kopp, W.C. *et al*. (1992) Polyinosinic-polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose in combination with interleukin 2 in patients with cancer: clinical and immunological effects. *Cancer Res.*, **52** (11), 3005–3010.
[51] Alfaro, C., Perez-Gracia, J.L., Suarez, N. *et al*. (2011) Pilot Clinical Trial of Type 1 Dendritic Cells Loaded with Autologous Tumor Lysates Combined with GM-CSF, Pegylated IFN, and Cyclophosphamide for Metastatic Cancer Patients. *J. Immunol. (Baltimore, MD)*, **187** (11), 6130–6142.
[52] Morse, M.A., Chapman, R., Powderly, J. *et al*. (2011) Phase I Study Utilizing a Novel Antigen-Presenting Cell-Targeted Vaccine with Toll-like Receptor Stimulation to Induce Immunity to Self-Antigens in Cancer Patients. *Clin. Cancer Res.*, **17** (14), 4844–4853.
[53] Lin, Q., Li, W., Lin, Y. *et al*. (2012) Toll-like Receptor 3 Ligand Polyinosinic:polycytidylic Acid Promotes Wound Healing in Human and Murine Skin. *J. Invest. Dermatol.*, **132** (8)**,** 2085–2092.
[54] Hafner, A., Corthésy, B. and Merkle, H. (2013) Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant. *Adv. Drug Deliv. Rev.*, **65** (10), 1386–1399.
[55] Forte, G., Rega, A., Morello, S. *et al.* (2012) Polyinosinic-polycytidylic acid limits tumor outgrowth in a mouse model of metastatic lung cancer. *J. Immunol.*, **188** (11), 5357–5364.
[56] Lin, B., Zhao, H., Fan, J. *et al*. (2014) B16 cell lysates plus polyinosinic-cytidylic acid effectively eradicate melanoma in a mouse model by acting as a prophylactic vaccine. *Mol. Med, Rep*., **10** (2), 911–916.
[57] Huang, C., Zhang, Q., Guo, X.K. *et al.* (2014) Porcine reproductive and respiratory syndrome virus nonstructural protein 4 antagonizes beta interferon expression by targeting the NF-kB essential modulator. *J. Virol.*, **88** (18), 10934–10945.
[58] Meier, W., Husmann, R., Schnitzlein, W. *et al*. (2004) Cytokines and synthetic double-stranded RNA augment the T helper 1 immune response of swine to porcine reproductive and respiratory syndrome virus. *Vet. Immunol. Immunopathol.*, **102** (3), 299–314.
[59] Verdijk, Rm., Mutis, T., Esendam, B. *et al*. (1999) Polyriboinosinic polyribocytidylic acid (poly(I:C)) induces stable maturation of functionally active human dendritic cells. *J. Immunol.*, **163** (1), 57–61.
[60] Caskey, M., Lefebvre, F., Filali-Mouhim, A. *et al*. (2011) Synthetic double-stranded RNA induces innate immune responses similar to a live viral vaccine in humans. *J. Exp. Med.*, **208** (12), 2357–2366.
[61] Trumpfheller, C., Caskey, M., Nchinda, G. *et al*. (2008) The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. *Proc. Natl. Acad. Sci. USA*, **105** (7), 2574–2579.
[62] Resaee, F., Meednu, N., Emo, J.A. *et al*. (2011) Polyinosinic:polycytidylic acid induces protein kinase D-dependent disassembly of apical junctions and barrier dysfunction in airway epithelial cells. *J. Allergy Clin. Immunol.*, **128** (6), 1216–1224.
[63] Becker, K.C. and Becker, R.C. (2006) Nucleic acid aptamers as adjuncts to vaccine development. *Curr. Opin. Mol. Ther.*, **8** (2), 122–129.
[64] Wengerter, B.C., Katakowski, J.A., Rosenberg, J.M. *et al*. (2014) Aptamer-targeted antigen delivery. *Mol. Ther.*, **22** (7), 1375–1387.
[65] Avci-Adali, M., Steinle, H., Michel, T. *et al*. (2013) Potential capacity of aptamers to trigger immune activation in human blood. *PLoS One*, **8** (7), e68810.
[66] Pratico, E.D., Sullenger, B.A. and Nair, S.K. (2013) Identification and characterization of an agonistic aptamer against the T cell costimulatory receptor, OX40. *Nucleic Acid Ther.*, **23** (1), 35–43.

[67] Steiner, J.M., Sartor, M., Sanchez, A.B. *et al*. (2010) DeNAno: Selectable deoxyribonucleic acid nanoparticle libraries. *J. Biotechnol.*, **145** (4), 330–333.
[68] Dollins, C.M., Nair, S., Boczkowski, D. *et al*. (2008) Assembling OX40 aptamers on a molecular scaffold to create a receptor-activating aptamer. *Chem. Biol.*, **15** (7), 675–682.
[69] Hui, Y., Shan, L., Lin-Fu, Z. and Jian-Hua, Z. (2007) Selection of DNA aptamers against DC-SIGN protein. *Mol. Cell. Biochem.*, **306** (1–2), 71–77.
[70] Tang, L., Yang, X., Dobrucki, L.W. *et al*. (2012) Aptamer-functionalized, ultra-small, monodisperse silica nanoconjugates for targeted dual-modal imaging of lymph nodes with metastatic tumors. *Angew. Chem., Int. Ed. Engl*., **51** (51), 12721–12726.
[71] Pan, Q., Wang, Q., Sun, X. *et al*. (2014) Aptamer against mannose-capped lipoarabinomannan inhibits virulent Mycobacterium tuberculosis infection in mice and rhesus monkeys. *Mol. Ther.*, **22 (**5**),** 940–951.
[72] Applicant: POWDERJECT RES LTD (2006) Nucleic Acid Coated Particles. EC: C12N15/89B; A61K47/48W8B; (+1) IPC: C12N15/86; A61K48/00; C12N15/87 (+3) Publication info: US Patent 2006153804, A1 – filed July 13, 2006.
[73] Applicant: POWDERJECT RES LTD (GB) (2005) Nucleic Acid Coated Particles. EC: A61K9/16K2; A61K48/00B IPC: A61K9/16; A61K48/00; A61K9/16 (+4) Publication info: European Patent 1545593, A1 – filed June 29, 2005.
[74] Applicant: POWDERJECT RES LTD (GB) (2006) Nucleic Acid Coated Particles. EC: A61K9/16K2; A61K48/00B IPC: A61K9/16; A61K48/00; A61K9/16 (+4) Publication info: British Patent GB 0508549D, D0 – filed June 1, 2005. GB 2410497, A – filed August 3, 2005. GB 2410497, B – filed Nov. 22, 2006.
[75] Applicant: POWDERJECT RES LTD (GB) (2004) Nucleic Acid Coated Particles. (+1) EC: A61K9/16K2; A61K48/00B IPC: A61K9/16; A61K48/00; A61K9/16 (+4) Publication info: World Intellectual Property Organization Patent WO 2004028560, A1 – filed April 8, 2004.
[76] Applicant: POWDERJECT RES LTD (GB) (2005) Nucleic Acid Coated Particles. EC: A61K9/16K2; A61K48/00B IPC: A61K9/16; A61K48/00; A61K9/16 (+2) Publication info: Chinese Patent CN 1694721, A – filed Nov. 9, 2005.
[77] Applicant: POWDERJECT RESEARCH LTD (2007) Nucleic Acid Coated Particles. EC: A61K9/16K2; A61K48/00B IPC: A61K9/16; A61K48/00; A61K (+2) Publication info: Hong Kong Patent HK 1073797, A1 – filed May 11, 2007.
[78] Applicant: POWDERJECT RESEARCH LTD (2006) Nucleic Acid Coated Particles. EC: A61K9/16K2; A61K48/00B IPC: A61K9/18; A61K9/16; A61K38/00 (+13) Publication info: Japanese Patent JP 2006503063T, T – filed Jan. 26, 2006.
[79] Applicant: POWDERJECT RES LTD (GB) (2005) Nucleic Acid Coated Particles. EC: A61K9/16K2; A61K48/00B IPC: A61K9/16; A61K48/00; A61K9/16 (+4) Publication info: Korean Patent KR 20050070008, A – filed July 5, 2005.
[80] Applicant: POWDERJECT RES LTD (2004) Nucleic Acid Coated Particles. EC: A61K9/16K2; A61K48/00B IPC: A61K9/16; A61K48/00; A61K9/16 (+4) Publication info: Australian Patent AU 2003269213, A1 – filed April 19, 2004.
[81] Applicant: POWDERJECT RES LTD (GB) (2005) Nucleic Acid Coated Particles. EC: A61K9/16K2; A61K48/00B IPC: A61K9/16; A61K48/00; A61K9/16 (+4) Publication info: Brazilian Patent BR 0314751, A – filed July 26, 2005.
[82] Applicant: POWDERJECT RES LTD (GB) (2004) Nucleic Acid Coated Particles. EC: A61K9/16K2; A61K48/00B IPC: A61K9/16; A61K48/00; A61K9/16 (+4) Publication info: Canadian Patent CA 2500215, A1 – filed April 8, 2004.
[83] Applicant: POWDERJECT RES LTD (GB) (2005) Nucleic Acid Coated Particles. EC: A61K9/16K2; A61K48/00B IPC: A61K9/16; A61K48/00; A61K9/16 (+4) Publication info: Mexican Patent MXPA 05003222, A – filed July 26, 2005.
[84] Applicant: POWDERJECT RES LTD (GB) (2005) Nucleic Acid Coated Particles. EC: A61K9/16K2; 61K48/00B IPC: A61K9/16; A61K48/00; A61K9/16 (+4) Publication info: Polish Patent PL 375782, A1 – filed Dec. 12, 2005.
[85] Lin, Z., Monteiro-Riviere, N.A., and Riviere, J.E. (2015) Pharmacokinetics of metallic nanoparticles. *Wiley Interdisciplinart Rev.: Nanomed. Nanobiotechnol*., **7** (2), 189–217.

[86] DeLong, R., Mitchell, J.A., Morris, R.T. *et al*. Enzyme and cancer cell selectivity of nanoparticles: inhibition of 3-D metastatic phenotype and experimental melanoma by zinc oxide. *J. Biomed. Nanotechnol*. (submitted).

[87] DeLong, R., Risor, A., Kanomata, M. *et al.* (2012) Characterization of biomolecular nanoconjugates by high-throughput delivery and spectroscopic difference. *Nanomedicine (London)*, **7** (12), 1851–1862.

[88] Dean, J.M. and DeLong, R.K. (2015) A high throughput screening assay for the functional delivery of splice switching oligonucleotides in human melanoma cells. *Methods Mol. Biol.*, **1297**, 187–196.

[89] Lee, Y.R., Lee, Y.H., Im, S.A. et al. (2010) Biodegradable nanoparticles containing TLR3 or TLR9 agonists together with antigen enhance MHC-restricted presentation of the antigen. *Arch. Pharm. Res.*, **33** (11), 1859–1866.

[90] Medberry, P., Dennis, S., Van Hecke, T. and DeLong, R.K. (2004) pDNA bioparticles: comparative heterogeneity, surface, binding, and activity analyses. *Biochem. Biophys. Res. Commun.*, **319** (2), 426–432.

[91] Stone, G.W., Barzee, S., Snarsky, V. *et al*. (2009) Nanoparticle-delivered multimeric soluble CD40L DNA combined with Toll-Like Receptor agonists as a treatment for melanoma. *PLoS One*, **4** (10), e7334.

[92] Papis, E., Rossi, F., Raspanti, M. *et al*. (2009) Engineered cobalt oxide nanoparticles readily enter cells. *Toxicol. Lett.*, **189** (3), 253–259.

[93] Manani, G.N., Spidle, R.T., Bhaumik, A. *et al*. (2016) Novel aqueous fabrication and characterization of gold coated cobalt nanoparticles. *Curr. Bionanotechnol.*, **1** (1), 1–6.

[94] Mohamed Basith, N., Vijaya, J., Kennedy, L. *et al.* (2014) Co-doped ZnO nanoparticles: structural, morphological, optical, magnetic and antibacterial studies. *J. Mater. Sci. Technol.*, **30** (11), 1108–1111.

[95] Shu, D., Shu, Y., Haque, F. *et al*. (2011) Thermodynamically stable RNA three-way junction for constructing multifunctional nanoparticles for delivery of therapeutics. *Nat. Nanotechnol.*, **6** (10), 658–667.

[96] Khisamutdinov, E., Li, H., Jasinski, D. *et al*. (2014) Enhancing immunomodulation on innate immunity by shape transition among RNA triangle, square and pentagon nanovehicles. *Nucleic Acids Res.*, **42** (15), 9996–10004.

[97] Ulmer, J.B., Mason, P.W., Geall, A. and Mandl, C.W. (2012) RNA-based vaccines. *Vaccine*, **30** (30), 4414–4418.

[98] Vacchelli, E., Galluzzi, L., Eggermont, A. *et al*. (2012) Trial watch: FDA-approved Toll-like receptor agonists for cancer therapy. *Oncoimmunology*, **1** (6), 894–907.

[99] Li, X., Zhao, Q. and Qui, L. (2013) Smart ligand: aptamer-mediated targeted delivery of chemotherapeutic drugs and siRNA for cancer therapy. *J. Control. Release*, **171** (2), 152–162.

[100] Herrmann, A., Priceman, S.J., Kujawski, M. *et al*. (2014) CTLA4 aptamer delivers STAT3 siRNA to tumor-associated and malignant T cells. *J. Clin. Invest.*, **124** (7), 2977–2987.

[101] Hilleman, M. (2003) Critical overview and outlook: pathogenesis, prevention, and treatment of hepatitis and hepatocarcinoma caused by hepatitis B virus. *Vaccine*, **21** (32), 4626–4649.

16

Local Pulmonary Host-Directed Therapies for Tuberculosis via Aerosol Delivery

Mercedes Gonzalez-Juarrero

Mycobacteria Research Laboratories, Microbiology Immunology and Pathology, Colorado State University, Fort Collins, CO, 80523, USA

16.1 Introduction

Despite being an ancient disease, tuberculosis (TB) remains one of the three major disease killers worldwide, alongside AIDS and malaria. The lack of availability of an effective protective vaccine and the lack of sterilizing drugs makes control of TB very difficult. At present, 1.5 million people die of TB every year. Current chemotherapy against this bacterial infection is lengthy and the low patient compliance has resulted in an elevated number of multidrug-resistant (MDR-TB) and extensively drug-resistant (XDR-TB) forms of TB. According to the World health Organization (WHO), to control TB it is essential to develop shorter, effective and well-tolerated treatments for latent TB infection, as this is the only way to reduce the development of resistance [1]. To develop new and more effective therapies capable of reaching rapid sterilization of the lungs it is necessary to address the following scientific questions; (1) why is current multidrug therapy for TB not capable of rapidly sterilizing the lungs? (2) If the immune system is capable of eliciting strong pulmonary immunity against these bacilli, why is it not capable of reaching sterilization of the lungs? (3) Do host immunity and TB chemotherapy synergize or antagonize each other? One approach to developing new therapeutic approaches for TB considers a combination

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

of current anti-TB chemotherapies with host-directed therapies (HDTs) delivered via aerosol. In this chapter we discuss previous attempts in the history of TB research to deliver HDTs via aerosols as well as potential pulmonary immune targets to develop further aerosols of HDTs.

16.1.1 Tuberculosis Disease and Control

TB remains one of the world's deadliest communicable diseases. According to the WHO Global Report of 2014 [1], in 2013 there were an estimated 9.0 million people suffering from TB. However, intensive efforts by international agencies and national campaigns of TB prevention, care and control are slowly controlling the incidence of new cases of the disease. The 2015 Millennium Development Goal (MDG) of halting and reversing TB incidence has been achieved globally. Worldwide, between 2000 and 2013 TB incidence fell at an average rate of about 1.5% per year. However, despite reaching the 2015 MDG, there are major concerns about the epidemiological control of TB disease globally because there is no improvement in the outcome of (MDR-TB) treatment and the number of TB cases with poor treatment outcomes is rising. Globally the success rate for MDR-TB in 2013 was only 48%, mostly because of the lack of appropriate health systems, lack of effective regimens and lack of patient compliance. As a result, the WHO has identified five priority actions to control the MDR-TB epidemic. These are: (1) high-quality treatment of drug-susceptible TB to prevent development of new strains of MDR-TB; (2) expansion of rapid testing and detection of MDR-TB cases; (3) immediate access to quality care; (4) infection control; and (5) *research to develop new diagnostics, drugs and treatment regimens*. The WHO post-2015 global TB strategy includes "*Intensified Research and Innovation within two essential and complementary areas: (1) Discovery, development and rapid uptake of new tools, interventions and strategies; and (2) Research to optimize implementation and impact*" [1].

16.1.2 Chemotherapy and Host Immunity to Tuberculosis

The lengthiness of chemotherapy treatment is a big limitation to the quick and efficient control of the global TB epidemic. Today new cases of drug-susceptible TB are still treated with a six-month multidrug-regimen of four first-line drugs; isoniazid, rifampicin, ethambutol and pyrazinamide. Treatment for MDR-TB, defined as resistance to isoniazid and rifampicin (the two most powerful anti-TB drugs), requires more expensive and toxic drugs administered by injection during at least twenty months. To develop new therapies it is important first to analyze the reason(s) behind the time requirement necessary for multidrug TB therapy to reach sterilization: (1) From the chemotherapeutic field perspective this question was addressed in several studies using experimental animal models to examine the killing kinetics of *Mycobacterium tuberculosis* (*M. tuberculosis* and causative agent of most cases of pulmonary TB in humans), during administration of standard TB chemotherapy [2–4]. These studies showed that the standard multidrug TB chemotherapy is capable of rapidly reducing the drug-sensitive pulmonary bacterial load (1–2 $\log_{10}$ reduction) during the first two weeks of treatment but the bacilli remaining after this period of treatment (drug tolerant and less than 1% of total lung burden) require 3–4 additional months of treatment to be eliminated. Importantly, in human TB patients there is a similar trend of response to standard TB multidrug chemotherapy [2–4]. Thus, TB treatment requires

long-term multidrug regimens because of a small persistent population of drug-tolerant bacilli. (2) From the immunological point of view the overall host immune response to *M. tuberculosis* in the lungs also limits the efficacy of anti-TB chemotherapy and interferes with current chemotherapy. Currently, the antibiotic mechanisms of most TB drugs primarily function to inhibit bacterial protein synthesis, DNA damage and cell-wall biosynthesis and therefore are primarily effective against metabolically active and replicating bacilli. However - and as explained below - during chronic pulmonary infections most bacteria in the lungs are found in a low replicative/metabolic stage within granuloma lesions. Consequently, anti-TB drugs are not highly effective against low replicative/metabolic bacilli escaping from the host immunity. Thus, one approach to improve current treatment regimens is to develop HDTs as adjunct therapies to current TB chemotherapy to shorten the length of treatment for TB or to treat drug-resistant TB cases. It is suggested that HDTs can be used to reactivate the bacilli metabolic/replicative stage within the granuloma, thus the bacilli can be more effectively targeted by antibiotics. Alternatively the HDTs can be directed to enhance the host's bactericidal capacity while helping to eliminate those few bacilli not responding to anti-TB chemotherapy.

16.1.3 Aerosol Delivery of Host-Directed Therapies for Tuberculosis Treatment

Tuberculosis is primarily a disease of the lungs and thus the use of aerosols to deliver TB drugs directly to the lungs is a logical approach. Advantages of using aerosols of HDT for TB are delivery of high concentrations of the drugs at the site of infection, therefore reducing systemic concentrations and systemic side effects. Although successful attempts to deliver aerosol antibiotics to TB patients were initiated in 1950 [5, 6], the TB research field has not invested heavily in the development of this type of therapy although the last two decades have witnessed extensive research and development of successful inhalational therapy for other pulmonary diseases [7] and thus the lessons learnt from other inhalational therapies can also be applied to advance the field of TB treatment.

16.2 Lung Immunity to Pulmonary *M. tuberculosis* Infection

16.2.1 Overview

By nature the lung environment tends to be hypo-responsive to the continuing circulation of particles entering through the airways, including microorganisms. In the lungs, multiple immunosuppressive mechanisms down-regulate many antigenic stimuli to prevent potentially destructive immune and inflammatory reactions. In many instances the recruitment of immune cells (inflammation) along with potent anti-inflammatory mechanisms co-exist. This scenario creates an ideal environment for long-term survival of some microorganisms, including the *M. tuberculosis* bacilli. Thus, when the lungs are exposed to *M. tuberculosis*, if the bacteria are not eliminated by innate mechanisms of immunity then the bacteria eventually encounter phagocytic cells in the alveolus known as alveolar macrophage (AM) and immature dendritic cells (DCs). Within these phagocytes, the bacteria are capable of establishing a niche for survival and replication by subverting fusion of the phagosome with the lysosomes [8]. Meanwhile, the bactericidal and bacteriostatic effect of the immune system is initiated at the draining lymphoid tissue via production of *M. tuberculosis*

antigen-specific T cells with a phenotype Th1 capable of producing IL-12, IL-23, IFNγ, TNFα, and IL-17 cytokines (IL = interleukin; IFN = interferon; TNF = tumor necrosis factor). Chief among these cytokines is IFNγ, a cytokine that ultimately performs the bactericidal/bacteriostatic effect of the adaptive immunity. However, when compared with pulmonary infection with other pathogens (e.g., influenza virus) the adaptive response developed in response to *M. tuberculosis* infection is a relatively slow process. As a result, by the time the Th1 cells reach the lungs (inflammatory process) and deliver IFNγ to the site of the infection, the *M. tuberculosis* bacilli have increased in population size within phagocytic cells and have become refractory to the bactericidal effect of the Th1 response. This inflammatory process develops further into the formation of a granuloma lesion, which is capable only of providing bacteriostatic effects over long periods of time but is incapable of sterilizing the lungs. At this time the pulmonary *M. tuberculosis* infection enters a chronic stage during which the bacteria are capable of remaining within the granuloma lesion in a low replicative/low metabolic stage for long periods of time. Also, during the long chronic stage of the infection, the bacteria are capable of maneuvering the anti-inflammatory and immunosuppressive responses of the lungs to their own benefit. If successful the bacteria can then replicate more rapidly with subsequent activation of TB disease. In summary, a continuous balance between inflammatory and anti-inflammatory responses along with balanced bacteriostatic and immunosuppressive mechanisms protect the host from disease despite being infected with *M. tuberculosis* for long periods of time.

16.2.2 Influence of Lung Alveoli Environment on Bacilli Survival and its Impact on Tuberculosis Chemotherapy

The limited bactericidal effect derived from the host immunity against *M. tuberculosis* can be further traced to the anti-inflammatory and immune suppressive (M2 type) or pro-inflammatory and bactericidal (M1 type) properties ascribed to AMs and DCs within the lung alveoli. Thus, AMs and DCs are found in the lung alveoli at different stages of differentiation [9] and in different activation stages (M1 or M2). They serve to limit inflammation and minimize lung injury (M2 type) but they are also sentinels against microorganisms entering the airways (M1 type) [10–12]. The immunological function of these cells in each alveolus appears to also be shaped by the type of surfactants lining in the lung alveolus [11, 13]. Thus the progression and final outcome of the initial infection with *M. tuberculosis* bacilli is dependent on the type (M1 or M2) and stage of differentiation of the AM and DC that the bacteria encounter within the alveolus. It is believed that each alveolus provides a unique immune environment to each bacillus entering the airways. The latter explains the fact that the type-activation stage of the infected cell encountering the bacilli at the alveolus drives initiation of the granuloma formation under different types of inflammatory/anti-inflammatory responses, leading to different levels bactericidal/bacteriostatic capacity in each granuloma. As a result a wide variety of granulomatous lesion types can be found in the same host [14]. Thus, differences in the immune environment of the alveolus, type of AMs and DCs in the alveolus and ultimately granulomas most likely influence the location, metabolism and state of the bacilli during infection. Eventually all of these factors determine the drug responsiveness by the bacilli and overall efficacy of the drug therapy in the infected host.

16.2.3 Potential Targets for Host-Directed Therapy

16.2.3.1 Potential Targets for Host-Directed Therapy in Macrophage Cells

The granuloma lesion requires continuous recruitment of different types of monocytes, macrophages and DCs in addition to neutrophils and lymphocytes. The main function of these lesions is to restrain the bacilli growth while at the same time limiting inflammation and lung tissue damage via anti-inflammatory and tissue repair-remodeling mechanisms, respectively. The end result of these activities leads to microenvironments of macrophage and lymphocytes subsets engaging in anti-inflammatory or pro-inflammatory processes. Such microenvironments of macrophages within the granuloma are classified as anti-inflammatory and tissue repair-remodeling if the macrophages express the enzyme arginase 1 (Arg1) [15]. On the other hand, the classically activated pro-inflammatory macrophages that engage in bactericidal activity are characterized by expression of [inducible] nitric oxide (NO) synthase (NOS-2). It is important to note that NOS-2 and Arg-1 use the same substrate, L-arginine, and thereby increased consumption of L-arginine by one of the enzymes depletes availability of the substrate to the other enzyme, resulting in diminution/inhibition of its activity. A similar axis is the inflammatory–anti-inflammatory balance of macrophages within the tuberculous granuloma that has been described recently for eicosanoids, in which two enzymes feed on the same substrate [16, 17]. Eicosanoids are lipid mediators derived from arachidonic acid (AA) metabolism. Two classes of enzymes, the cyclooxygenases (COX) and lipoxygenases (LO), compete for the AA substrate to generate COX-1 and two dependent prostaglandins (PGE2) or in an opposing pathway 5-lipoxygenase (5-LO)-dependent lipoxins and leukotrienes. Based on susceptibility studies in the murine infection model, a concept similar to the Arg1/iNOS axis has emerged for the COX/5-LO axis. PGE2 confers resistance against *M. tuberculosis* infection, while products of the 5-LO axis, in particularly lipoxin A4 (LXA4), promote bacterial growth. In both pathways the enzymatic activities of NOS-2 and COX-2 limit *M. tuberculosis* growth while overexpression of Arg1 and/or 5-LO enzymes within the granuloma tilts the anti-inflammatory–inflammatory balance towards bacterial growth. These two enzymatic axes of control/susceptibility for *M. tuberculosis* infection are potential targets for HDT. Therapeutic intervention to enhance NOS-2 and/or COX-2 while reducing expression of Arg 1 and/or 5-LO could potentially drive the lung immune environment towards improving the host's bactericidal capacity. Indeed a recent study [16, 17] has supported the notion that direct administration of PGE2 or inhibition of the 5-LO with the already FDA-approved drug Zileuton (an inhibitor of 5-LO) can benefit the outcome of acute and chronic *M. tuberculosis* infections.

16.2.3.2 Potential Targets for Host-Directed Therapy in T Cells

The inflammatory–anti-inflammatory nature of the granuloma is also determined by the nature and progression of lymphoid cell populations within the granuloma. Granulomas present a dynamic change in the composition of Th1 and Th2 or T regulatory (Treg) cells during the course of the infection [18, 19]. Years ago, it was assumed that during a chronic *M. tuberculosis* infection the Th1 cells reaching the lungs were fully capacitated to deliver their IFNγ load and were capable of activating macrophages to eliminate *M. tuberculosis*. However, this appeared to be an over-simplification of the overall immunity against

M. tuberculosis infection. Several experimental studies using animal models of TB are demonstrating [20, 21] that increasing the Th1 response in the lungs via increased expression of CD4 T cells with Th1 phenotype and increased expression of the IFNγ cytokine does not necessary lead to increased bactericidal activity. These studies suggest that along with an increase in the Th1 immune response there are also counter-regulatory anti-inflammatory responses that limit the bactericidal effect. As discussed below, therapeutic administration of IFNγ aiming to increase the bactericidal effect of the Th1 responses in the lungs seems like a logical approach. However, clinical studies have shown this effect is transient and limited.

An alternative approach to modulate the T cell responses during TB is to target T regulatory cells in the granuloma. In this regard, it has been shown [19, 22, 23] that T regulatory cells infiltrate the granuloma and are capable of counteracting the Th1 responses via expression of the transcription factor FoxP3 and CTLA-4 and PD-1 antigens [23, 24]. *In vivo* and systemic CTLA-4 blockage enhanced the immune response but did not lead to enhanced clearance of the infection [25]. Current clinical studies in the cancer field are showing greatly successful tumor remission with systemic therapies targeting the Treg via blockage of CTLA-4 and PD-1 antigens in T cells [26]. Thus, because the granuloma and tumor immune environments are very similar and driven by the outstanding success of tumor remission in the cancer field with these therapies, local and pulmonary blockage of CTLA-4 and PD-1 positive cells (via aerosol) could potentially enhance the Th1 responses and be therapeutic targets against TB.

16.2.3.3 Potential Targets for Host-Directed Therapy Targeting Cytokine Expression

Early clinical and preclinical studies have shown that successful maintenance of the chronic stage of infection and the metabolic and replicative activity of the bacilli within the granuloma are also dependent on the balance between inflammatory and anti-inflammatory cytokines [19, 22]. In this regard it is well established that the containment of *M. tuberculosis* growth within the granuloma requires a continuous expression of Th1 cytokines such as IL-12, IFNγ and TNFα at all times. If any of these cytokines are absent or blocked, TB disease reactivates [27]. Thus the counter-balance of IL-12/IFNγ/TNFα cytokines is an essential pathway for adequate regulation of bactericidal effect in the host as well as long-term containment of the bacilli within the granuloma lesions. These cytokines have already been targeted for therapeutic intervention either by therapeutic administration of IFNγ to augment the bactericidal effect of the Th1 response or by inhibition of TNFα to suppress excessive expression of this cytokine.

Other effector cytokines that play important roles in *M. tuberculosis* infection are the IL-1 family members, IL-1α and IL-1β and the Type 1 IFNs. Both species of IL-1 signal through the same IL-1 receptor and are independently required for host resistance against an infection with *M. tuberculosis*, but overexpression of the IL-1 production can also lead to tissue damage and pathology. On the other hand Type 1 IFNs, both IFNα and IFNβ, are associated with suppression of Th1 responses, and thus overexpression of the Type 1 IFNs *in vivo* appears to promote bacterial growth and survival. Immune pathways mediated by IL-1 and Type 1 IFNs have opposing roles in host resistance to infection as production of both IL-1α and IL-1β is inhibited by Type 1 IFNs in human and experimental infections. Together the IL-1 and Type 1 IFNs share an important counter-regulatory axis that could be utilized as a target for HDT intervention and these are currently under investigation [16, 17, 28, 29].

On the other hand IL-10 and transforming growth factor beta 1 (TGFβ1) are suppressive cytokines in the lungs that promote fibrosis, healing and anti-inflammatory responses [30–36]. These immunosuppressive cytokines appear to also be potent inhibitors of the inflammatory Th1 response in the granuloma, but they are activated late in the immune response when compared with other cytokines. Thus, during the chronic stage of infection the continuous production of immunosuppressive cytokines can override the protective activity of Th1 cytokines, leading to reactivation of bacterial growth and active TB disease. In this scenario, it is believed that therapeutic intervention to overcome excessive production of anti-inflammatory/immunosuppressive cytokines [or inhibition of their downstream activities] in the lungs during the chronic stage of infection with *M. tuberculosis* could also be a potential target for HDT aiming to rescue the bactericidal effect of the Th1 response.

16.3 Host-Directed Therapies

16.3.1 Previous Studies via Systemic Administration of Host-Directed Therapies

Several articles are available that provide an historical review of previous attempts to use HDTs for TB via systemic administration [17, 37–39]. As mentioned above, an early hypothesis was that increasing the Th1 responses should improve the bactericidal capacity in the host. Thus, previous HDTs attempts for TB were aimed at increasing immune killing of the persisting bacilli by enhancing the Th1 responses (e.g., through systemic administration of IL-2 [40, 41], IFNγ [42–45] or IFNα [46] cytokines to patients with TB). Overall these studies provided some benefits but they did not reach sterilization or shorten the duration of chemotherapy and any positive effects were reversed upon cessation of treatment. When those studies were developed in the1980s–2000s [42–45], the concept of the inflammatory/anti-inflammatory nature of the granuloma and the role of Th1 cytokines being counterbalanced by immunosuppression and anti-inflammatory responses was not as well developed as it is now. Thus, one possible explanation for the limited and temporary bactericidal effect observed in the TB clinics with this approach is that over time therapeutic administration of IL-2 or IFNγ cytokines leads to activation of the anti-inflammatory/immunosuppressive mechanisms to limit potential inflammation and tissue damage, which subsequently reduced the therapeutic bactericidal effect.

Targeting the inflammatory response (e.g., TNF and its pathways) has met with more success. As mentioned above, a threshold of TNFα production (or Th1 cytokines in general) is needed to contain reactivation of the tubercle bacilli and to maintain the integrity of the granuloma; however, excessive production of TNFα cytokine is also responsible for most of the serious clinical symptoms [night sweats, fever, progressive weight loss] associated with active TB disease. Excessive production of TNFα is also responsible for extensive necrosis at the lesion site and for cavitation [47]. Thus, it has been reasoned that targeting excess of TNFα by using immunotherapy may enhance anti-TB chemotherapy. Thalidomide is a drug that reduces TNFα levels via inhibition of phosphodiesterase (PDE) in patients with pulmonary TB and improves treatment outcomes but has serious side effects. The Thalidomide analogs targeting the PDE4, viz. CC3052 and IMiD3, among others, are currently under evaluation in murine and rabbit models of TB disease [48–50]. The results indicate faster clearance of bacilli when given in conjunction with anti-TB chemotherapy [42–45]. However, results from the same group using anti-TNFα antibodies to block excess

of TNFα in TB-infected mice resulted in uncontrolled growth of bacteria and had no synergistic effect with anti-TB chemotherapy. These dramatic differences highlight that targeting the same cytokine by different drugs can result in opposite therapeutic outcomes. Other TNFα blockers, such as infliximab (anti-TNFα antibody) or etanercept (a soluble TNFα receptor), reactivate TB disease when administered to patients with latent TB [51]. Regardless, in a study where human immunodeficiency virus (HIV)-infected patients with pulmonary TB received etanercept at the initiation of standard TB chemotherapy there was more rapid sputum conversion clearance in treated patients compared with those not receiving the TNFα blocker. This study suggested that anti-TNFα immunotherapy appears to reactivate the growth of the bacteria and accelerate the bactericidal efficacy of anti-TB chemotherapy in patients with active TB, demonstrating that modulation of the immune response as adjunct therapy to TB chemotherapy is a valid approach [51, 52].

As mentioned above, another approach to developing HDT for TB is to target immunosuppressive pathways. More specifically, these approaches target the over-expression of the TGFβ1 and IL-10 cytokines during the chronic infection with *M. tuberculosis*, because it is these pathways that control the persisting infectious state. Preliminary studies in the field of HDT for TB also targeted expression of the TGFβ1. It is known that TGFβ1 is highly expressed during TB infection of human and animal models. Moreover, its expression is higher in MDR-TB infection than during infection with drug-sensitive TB strains [53, 54]. Thus, it was reasoned that reducing the levels of TGFβ1 would improve TB chemotherapy because TGFβ1 is responsible for most of the fibrosis associated with TB and it is believed that reducing fibrosis might improve the access of TB drugs to the persistent bacilli. Several studies using the murine and guinea pig TB models of infection have been reported that target TGFβ1. Among those, a study showed that inhibitors of TGFβ1 in mice are useful therapeutic agents in combination with TB chemotherapy and significantly decreased lung fibrosis and bacillary load [55, 56]. However, this also leads to increased inflammatory responses in the lungs. In another study naturally occurring inhibitors of TGFβ1 such as decorin and latency-associated peptides were studied *in vitro* to reverse depressed T and monocytes cell functions in patients with TB [30]. In our experience the use of aerosols of small interfering RNA (siRNA) to transiently knock down expression of TGFβ1 in mice chronically infected with *M. tuberculosis* reduced bacterial load but also led to extensive exacerbation of the pulmonary inflammatory response. Overall in our laboratory it was concluded that targeting TGFβ1 is not a safe target for HDT (unpublished data).

On the other hand, as explained above, *M. tuberculosis* bacteria also benefit from an environment abundant in the immunosuppressive cytokine IL-10. This cytokine is a major down-modulator of both the Th1 function and its subsequent bactericidal effect and inflammation. The host generally up-regulates the expression of IL-10 cytokine late in the response to inflammation and infection with *M. tuberculosis*. In our experience and that of others, blocking the production of IL-10, its receptor or the signaling pathways during experimental infection with *M. tuberculosis* has resulted in decreased bacterial load and better control of the inflammatory response [57, 58]. To date, IL-10 signaling-pathway blockage during chronic infection with *M. tuberculosis* appears as a promising target for developing HDTs against TB, because unlike other candidates for HDT intervention (e.g., TNFα or TGFβ1) there is no apparent development of host-detrimental effects.

16.3.2 Previous Studies via Aerosol Delivery of Host-Directed Therapies

As observed in the introduction, the slow decline in TB incidence and the growing number of TB cases not responding to current TB chemotherapies is stressing the urgent need for new approaches to treat TB in terms of new drugs and improved methods of delivery. In this line of thinking and given that the primary route of *M. tuberculosis* infection is through the lungs, aerosol delivery of HDTs via inhalation either alone or in combination with TB chemotherapy has been studied. The major advantage of this approach is the delivery of high doses of the drugs at the site of infection while minimizing systemic toxicity and potential systemic development of autoimmunity. Although this approach has not been widely pursued in the history of TB research there is evidence from TB clinics and experimental models of TB that show that it is feasible to deliver drugs with HDT/antibiotic activity directly to the lung and induce changes in the course of a chronic *M. tuberculosis* infection. Furthermore, to date and contrary to what was previously thought, it is becoming widely accepted that the tuberculous granulomas are dynamic structures with active traffic of immune mediators which are capable of responding to changes in external signals. Specifically, it is well known that (1) systemic reduction of CD4 T cells in patients with latent TB [as in HIV-AIDS] leads to development of active TB. (2) Infusion of systemic antibodies that block TNFα in TB patients with rheumatoid arthritis interferes with the granuloma structure and can reactivate latent TB within weeks [51, 59, 60]. (3) Experimental studies with systemic injections of antibodies against CD4 or CD8 antigen depleted these cells from the granuloma [61]. (4) Most recently we demonstrated that administration of anti-TB drugs used to treat MDR-TB (spectinamides, amikacin, capreomycin) when administered directly to the lungs of mice with chronic infection of *M. tuberculosis* has almost equal or better (e.g., spectinamide 1599) efficacy than when administered systemically as injectables [62, 63]. All of this evidence indicates that the tuberculous granulomas are dynamic structures responding to changes in their external environment within the lungs. Therefore, the evidence suggests that aerosol administration of drugs with HDT activity can potentially steer the course of a chronic *M. tuberculosis* infection to the benefit of the host.

There are only a limited number of clinical and preclinical studies addressing aerosols of HDTs and for the most part the development of inhalational HDTs for TB is in its infancy. The first studies used aerosols of IFNγ aiming to enhance the bactericidal effect of the Th1 responses. Five studies, however, were performed directly in the TB clinics [45, 64] between 1998 and 2009 and none of the results derived from these clinical trials provided conclusive results. The sample sizes of these trials were not very large, which made it difficult to find meaningful effects of IFNγ from a single trial [45]. Despite this, the findings from a meta-analysis published recently in a systematic review [45] suggested that IFNγ, especially when administered by aerosol three times weekly, could be beneficial in pulmonary TB and had statistically beneficial effects on clearance of bacilli from sputum and chest radiographic. Similarly, aerosols of IFNα administered to MDR-TB patients [46, 65] demonstrated a slight improvement in bacterial clearance and sputum conversion. It is noteworthy that these clinical trials were developed without previous optimization and validation in inhalational preclinical studies. Thus, the potential use of inhalational therapy of IFNγ as HDT for TB could benefit from preclinical studies to optimize and validate this approach using modern formulations of IFNγ for aerosol delivery and newly developed aerosol animal models of TB.

At present the only vaccine available for TB is Bacille-Calmette–Guerin (BCG), which is administered by injection. BCG is almost 100 years old and its efficacy to prevent pulmonary TB in adults is highly variable; however, it protects from severe forms of TB disease in children and for this reason is widely used around the world. New vaccines and improved methods of delivery are urgently needed and thus vaccines are also being studied for their efficacy as HDTs and aerosol delivery. Aerosol vaccines could facilitate mass immunization campaigns by easing administration strategy compared with injection as there is no need for medically trained personal, and both cost and medical waste are reduced. There are clinical and preclinical studies on aerosol TB vaccines dating back as far as the 1950s [66, 67]. However, today aerosol TB vaccines have emerged as an important approach to improve efficacy of TB vaccines for TB prevention and treatment. The development of aerosol TB vaccines is being facilitated by lessons learnt from the process of developing an aerosol for measles vaccine through the WHO Measles vaccine project [66] and by the already available technology developed over decades of research on aerosol treatment of other pulmonary diseases such as asthma, chronic pulmonary disease, and cystic fibrosis [7]. Interest in this re-emerging approach in TB vaccine research is further emphasized by a recent workshop held at the National Institute of Allergy and Infectious Diseases on April 2014 entitled "Developing aerosol vaccines for *Mycobacterium tuberculosis*" [66].

The development of aerosol TB vaccines requires a good understanding of the mucosal immunity and preparation of new formulations of vaccines for aerosol delivery. With the purpose of enhancing pulmonary immunity and host antimicrobial capacity several antigen formulations and vaccines are currently being studied [68–72]. Recent advances in the spray-drying preparation and aerosol delivery have facilitated re-formulation of BCG vaccine using spray-dried, nanoparticle [69, 73], or liposome chitosan [74] and poly(d,l-lactide-co-glycolide)–poly(ethyleneimine) (PLGA–PEI) vehicles [68] specifically designed for aerosol delivery. These new re-formulated vaccines have been tested in mice and guinea pigs and have demonstrated capacity to enhance pulmonary antimicrobial activity against *M. tuberculosis* infection [69, 73]. Specifically, nanoparticle formulations and aerosol delivery of the BCG to guinea pigs subsequently challenged with virulent *M. tuberculosis* have been shown to reduce the bacterial burden and to improve lung pathology when compared with control groups receiving the standard parenteral BCG [69, 73]. Similarly, aerosol administration of the Rv1733c DNA vaccine formulated with PLGA–PEI nanoparticles to the lungs of chronically infected mice increased T cell proliferation and IFNγ production when compared with control groups receiving intramuscular vaccinations. These results confirm that PLGA–PEI nanoparticles are an efficient DNA vaccine-delivery system to enhance T cell responses through pulmonary delivery in a DNA prime or protein boost vaccine regimen [68]. Overall, several studies have indicated that pulmonary delivery of DNA vaccines against TB may provide an advantageous delivery route compared with intramuscular immunization, and that increased immunogenicity can be achieved by delivery of aerosols of DNA encapsulated in chitosan nanoparticles [68].

Immunogenicity of aerosol TB vaccines has also been studied in several preclinical studies using the non-human primates (NHP) TB animal models. Vaccination of NHP [Indian rhesus macaques] [75] via intradermal BCG administration and an aerosol boost with Aeras-402, an adenovirus-35 (Ad35) vector expressing mycobacterial antigens 85A/B (Ag85A/B) and TB10.4 resulted in a transient improvement of the CD4 and CD8 T cell

responses to stimulation with Ag85/B in the aerosol Ad35-Ag85/B-treated group compared with control groups. Overall, this study suggested that aerosol delivery of the adenovirus-vectored TB vaccine candidate generated a much more robust mucosal response than that obtained following delivery via injection [75] but no protection was observed after challenge with a low dose of *M. tuberculosis*. A repetition of this study to investigate additional variants after intradermal BCG vaccination and boosting via aerosol administration with viral-vectored Ad5 M72 or Ad-EAST-6/Ag85B is now underway using Chinese rhesus macaques [66].

In another study BCG-primed NHPs when boosted with intradermal or aerosol administration of the modified vaccinia Ankara-vectored/Ag 85A (MVA85A) TB vaccine candidate resulted in systemic and pulmonary boost of specific immune responses against Ag85A in peripheral blood mononuclear cells (PBMC) and in lung CD4 T cells [76]. The same MVA85A vaccine candidate was also tested in a Phase I clinical trial comparing intradermal and aerosol administration of the vaccine. The aerosol delivery via nebulizer was well tolerated and demonstrated strong antigen-specific IFNγ responses using both delivery routes of administration [76]. The continued support for development of aerosol administration of vaccines is emphasized by recent plans in the TB clinics to test mucosal immunity after aerosol or intranasal administration of Ad5-Ag85A or TB/FLU-04L vaccines, respectively. This vaccine was successfully evaluated in a Phase I trial that demonstrated no vaccine-related serious adverse events [1]. The TB/FLU-04L is a recombinant influenza vectored with strain A/Puerto Rico/8/34 (H1N1) expressing *M. tuberculosis* antigens Ag85A and ESAT- 6. A Phase I trial in BCG-vaccinated QuantiFERON TB-Gold negative healthy adult volunteers using intranasal administration was recently completed, and a Phase IIa trial is planned [1].

16.4 Limitations of Preclinical Studies to Develop Inhalational Host-Directed Therapies for Tuberculosis

Preclinical testing of the efficacy of aerosolized HDT drugs and ways of promoting lung deposition along with pharmacokinetics is a prerequisite for the future safe and efficient clinical use of aerosols of HDT for TB. Other chapters in this book have addressed the development of nanoparticles, liquid and dry-powder formulations and the use of animal models for TB. To advance the field of inhalational therapies for TB it is important to develop preclinical studies using animal models of TB. Towards this goal several groups in the USA and India have developed inhalational TB animal models using non-invasive procedures in mice and guinea pigs [6, 62]. These procedures allow for repeated administration (at intervals of minutes, hours or days) of host-directed drugs [e.g., siRNAs, peptide inhibitors, small molecules] [58, 77, 78] and anti-TB drugs [6, 62, 63]. In preparation for inhalational *in vivo* studies there are several factors that need to be taken into account. The most important factors are the size of the TB animal model (mice or guinea pigs) along with the cost and amount of drug available for the study. Thus, the development of aerosols of HDT using animal models for TB is limited by several logistic and economical factors; (1). The early stages of development of preclinical testing of only one drug require a large number (between 50 and 100) of animals per study. (2). Initial steps in the optimization and validation of inhalational research require relatively large amounts of drug which can be

expensive (3). These studies require technical staff with expertise to do aerosol treatments. (4). Treatments in animals have to be administered daily or several times per week for a period of two, four and sometimes eight weeks and have to be administered rapidly with precision and reproducibility.

Some inhalational animal models administer the drugs directly into the lungs while others use aerosol chambers or nose-only cones and the amount of drug needed per study can vary considerably. In this regard during the first steps of optimization and validation the use of a mouse model with direct and local deposition of the drug is recommended. In our laboratory we use the intrapulmonary aerosol administration in mice chronically infected with *M. tuberculosis* [58, 62, 63, 77, 78]. This procedure employs a specialized apparatus (the Penn-Century Microsprayer), and has the advantage of being a very quick procedure (30 seconds), reproducible and is very suitable for administering quantifiable doses to the deep lung. The intrapulmonary aerosol delivery allows for local and transient intervention aiming at reducing the amount of drug used per drug administration, toxicity and secondary effects. Other research groups use an inexpensive, home-made kit to make mice or guinea pigs inhale the drugs in a chamber or nose-only cone fitted to their nose [79, 80] where the drugs are aerosolized in dry-powder formulations prepared as described in other chapters of this book. This technique is excellent for allowing rapid, reproducible, painless and ambient-pressure inhalations to animals, but requires exceedingly large amounts of drugs to prepare and optimize the aerosols and lacks both the precision of dose delivery and the ability to avoid loss of drug in the mouth and upper airways. Thus, the intrapulmonary aerosol delivery is recommended in the earliest preclinical studies to determine the minimal concentration of drug providing bactericidal efficacy *in situ* in the lungs. Thereafter, this information can direct optimization of aerosols using chamber or nose-only cones as a next step in the optimization and validation process. In addition the size of each group of animals, concentration(s) of drugs to be tested and the number of doses to be administered along with the number of time points in the study need to be taken into account.

16.5 Preclinical Testing of Inhaled Small Interference RNA as Host-Directed Therapies for Tuberculosis

The small interference RNA (siRNA) mechanism has gained interest as a therapeutic approach because, in principle, any disease caused by, or greatly exacerbated by, the expression of a dominant gene can be treated with siRNA. As discussed above, several cytokines, enzymes, transcription factors, or in general overexpression of any immune mediator, can drive pathology or have an impact in the *M. tuberculosis* bacilli survival in the lungs, and these can be targeted by intrapulmonary aerosol administration of siRNA. A major challenge preventing widespread use of siRNA as a "gene therapy" has been achieving effective delivery to the target organ but fortunately our studies, and that of others, have shown siRNA uptake and biodistribution in the lung is very efficient. At present, our laboratory is investigating the use of siRNA as a tool to enhance the host's natural ability to clear the drug-tolerant bacilli and to study lung biology during an *M. tuberculosis* infection. Thus, transient and timely blocking of specific molecules (e.g., cytokines, cell-surface molecules, transcription factors) using siRNA during a *M. tuberculosis* pulmonary

infection provides a unique tool to study the lung biology of TB and to identify potential targets for HDT. In our studies intrapulmonary delivery of siRNAs aims at changing the immune environment of the granuloma and increasing the antimicrobial capacity via activation state of the macrophages within the granuloma to enhance their antimicrobial capacity while at the same time limiting the inflammatory responses. We have used siRNAs to target the expression of specific immunosuppressive cytokines [TGFβ1, IL-10, IL-6, granulocyte-macrophage colony stimulatory factor (GM-CSF) cytokines or signal transducer and activator of transcription 3 (STAT3)] because they play essential roles in facilitating the persistence of *M. tuberculosis* bacilli in the lungs [78, 81, 82]. In our experience using local immunotherapy delivery of siRNAs and cytokines directly into the lung of mice chronically infected with *M. tuberculosis* has served to demonstrate that it is possible to change the overall course of the pulmonary chronic *M. tuberculosis* infection and it therefore suggests a potential therapeutic approach [58, 77]. Using intrapulmonary aerosol delivery of siRNAs, any pathway and cytokines-chemokine/mechanisms or transcription factors implicated in the pathology of TB can be targeted. By identifying and studying the role of each molecule at specific time points and their contribution to the TB lung pathology, the identification and validation of new host targets for drug discovery [or FDA-approved and re-purposed drugs] will be facilitated and their potential use as aerosol HDT for TB can be fulfilled as suggested in this chapter.

Thus, as in all preclinical studies, the development of inhalational therapy using animal models of HDT for TB can be time consuming and resource intensive. In the early stages of identification of targets, the possible high cost in treatment and cost of drugs (e.g., recombinant cytokines, antibodies, siRNAs, peptides) targeting potential targets of immune pathways in the lungs are limitations to developing further this approach. However, the latter should not discourage research, as there is always the possibility for identification of existing drugs or design of new drugs with more practical and economical therapeutic value.

Acknowledgements

Support was provided by the NIH-NIAID AI-102210 and NIH-NIAID AI-105585.

References

[1] Zumla, A., George, A., Sharma, V. *et al*. (2015) The WHO 2014 Global tuberculosis report - further to go. *Lancet Global Health*, **3**(1), e10–e12.

[2] Franzblau, S.G., DeGroote, M.A., Cho, S.H. *et al*. (2012) Comprehensive analysis of methods used for the evaluation of compounds against *Mycobacterium tuberculosis*. *Tuberculosis*, **92**(6), 453–488.

[3] Lenaerts, A.J., Degroote, M.A. and Orme, I.M. (2008) Preclinical testing of new drugs for tuberculosis: current challenges. *Trends in Microbiology*, **16**(2), 48–54.

[4] Ordway, D.J., Shanley, C.A., Caraway, M.L. *et al*. (2010) Evaluation of standard chemotherapy in the guinea pig model of tuberculosis. *Antimicrobial Agents and Chemotherapy*, **54**(5), 1820–1833.

[5] Miller, J.B., Abramson, H.A. and Ratner, B. (1950) Aerosol streptomycin treatment of advanced pulmonary tuberculosis in children. *American Journal of Diseases of Children*, **80**(2), 207–237.

[6] Hickey, A.J., Misra, A. and Fourie, P.B. (2013) Dry powder antibiotic aerosol product development: inhaled therapy for tuberculosis. *Journal of Pharmaceutical Sciences*, **102**(11), 3900–3907.
[7] Hickey, A.J. (2013) Back to the future: inhaled drug products. *Journal of Pharmaceutical Sciences*, **102**(4), 1165–1172.
[8] Tan, S. and Russell, D.G. (2015) Trans-species communication in the *Mycobacterium tuberculosis*-infected macrophage. *Immunological Reviews*, **264**(1), 233–248.
[9] Gonzalez-Juarrero, M., Shim, T.S., Kipnis, A. *et al*. (2003) Dynamics of macrophage cell populations during murine pulmonary tuberculosis. *Journal of Immunology*, **171**(6), 3128–3135.
[10] Guirado, E., Schlesinger, L.S. and Kaplan, G. (2013) Macrophages in tuberculosis: friend or foe. *Seminars in Immunopathology*, **35**(5), 563–583.
[11] Rajaram, M.V., Ni, B., Dodd, C.E. and Schlesinger, L.S. (2014) Macrophage immunoregulatory pathways in tuberculosis. *Seminars in Immunology*, **26**(6), 471–485.
[12] Schlesinger, L.S. (1996) Role of mononuclear phagocytes in *M. tuberculosis* pathogenesis. *Journal of Investigative Medicine*, **44**(6), 312–323.
[13] Ferguson, J.S. and Schlesinger, L.S. (2000) Pulmonary surfactant in innate immunity and the pathogenesis of tuberculosis. *Tubercle and Lung Disease*, **80**(4–5), 173–184.
[14] Lenaerts, A., Barry, C.E., 3rd and Dartois, V. (2015) Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. *Immunological Reviews*, **264**(1), 288–307.
[15] Duque-Correa, M.A., Kuhl, A.A., Rodriguez, P.C. *et al*. (2014) Macrophage arginase-1 controls bacterial growth and pathology in hypoxic tuberculosis granulomas. *Proceedings of the National Academy of Sciences of the United States of America*, **111**(38), E4024–E4032.
[16] Mayer-Barber, K.D., Andrade, B.B., Oland, S.D. *et al*. (2014) Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature*, **511**(7507), 99–103.
[17] Mayer-Barber, K.D. and Sher, A. (2015) Cytokine and lipid mediator networks in tuberculosis. *Immunological Reviews*, **264**(1), 264–275.
[18] Shafiani, S., Tucker-Heard, G., Kariyone, A. *et al*. (2010) Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. *Journal of Experimental Medicine*, **207**(7), 1409–1420.
[19] Orme, I.M., Robinson, R.T. and Cooper, A.M. (2015) The balance between protective and pathogenic immune responses in the TB-infected lung. *Nature Immunology*, **16**(1), 57–63.
[20] Gonzalez-Juarrero, M., Turner, J., Basaraba, R.J. *et al*. (2002) Florid pulmonary inflammatory responses in mice vaccinated with Antigen-85 pulsed dendritic cells and challenged by aerosol with *Mycobacterium tuberculosis*. *Cellular Immunology*, **220**(1), 13–19.
[21] Olmos, S., Stukes, S. and Ernst, J.D. (2010) Ectopic activation of *Mycobacterium tuberculosis*-specific CD4+ T cells in lungs of CCR7-/- mice. *Journal of Immunology*, **184**(2), 895–901.
[22] Orme, I.M. and Basaraba, R.J. (2014) The formation of the granuloma in tuberculosis infection. *Seminars in Immunology*, **26**(6), 601–609.
[23] Cyktor, J.C., Carruthers, B., Beamer, G.L. and Turner, J. (2013) Clonal expansions of CD8+ T cells with IL-10 secreting capacity occur during chronic *Mycobacterium tuberculosis* infection. *PLoS One*, **8**(3), e58612.
[24] Henao-Tamayo, M., Irwin, S.M., Shang, S. *et al*. (2011) T lymphocyte surface expression of exhaustion markers as biomarkers of the efficacy of chemotherapy for tuberculosis. *Tuberculosis*, **91**(4), 308–313.
[25] Kirman, J., McCoy, K., Hook, S. *et al*. (1999) CTLA-4 blockade enhances the immune response induced by mycobacterial infection but does not lead to increased protection. *Infection and Immunity*, **67**(8), 3786–3792.
[26] Baksh, K. and Weber, J. (2015) Immune Checkpoint protein inhibition for cancer: preclinical justification for CTLA-4 and PD-1 blockade and new combinations. *Seminars in Oncology*, **42**(3), 363–377.
[27] Orme, I.M. and Cooper, A.M. (1999) Cytokine/chemokine cascades in immunity to tuberculosis. *Immunology Today*, **20**(7), 307–312.
[28] Mayer-Barber, K.D., Andrade, B.B., Barber, D.L. *et al*. (2011) Innate and adaptive interferons suppress IL-1alpha and IL-1beta production by distinct pulmonary myeloid subsets during *Mycobacterium tuberculosis* infection. *Immunity*, **35**(6), 1023–1034.

[29] Mayer-Barber, K.D., Barberm D.L., Shenderov, K. *et al*. (2010) Caspase-1 independent IL-1beta production is critical for host resistance to *Mycobacterium tuberculosis* and does not require TLR signaling in vivo. *Journal of Immunology*, **184**(7), 3326–3330.
[30] Hirsch, C.S., Yoneda, T., Averill, L. *et al*. (1994) Enhancement of intracellular growth of *Mycobacterium tuberculosis* in human monocytes by transforming growth factor-beta 1. *Journal of Infectious Diseases*, **170**(5), 1229–1237.
[31] Othieno, C., Hirsch, C.S., Hamilton, B.D. *et al*. (1999) Interaction of *Mycobacterium tuberculosis*-Induced Transforming Growth Factorβ1 and Interleukin-10. *Infection and Immunity*, **67**(11), 5730–5735.
[32] Toossi, Z. (1998) Adjunctive immunotherapy of tuberculosis. *Cytokines Cellular & Molecular Therapy*, **4**(2), 105–112.
[33] Ellner, J.J. (2010) Immunoregulation in TB: Observations and Implications. *Clinical and Translational Science*, **3**(1), 23–28.
[34] Turner, J., Gonzalez-Juarrero, M., Ellis, D.L. *et al*. (2002) *In Vivo* IL-10 production reactivates chronic pulmonary tuberculosis in C57BL/6 Mice. *Journal of Immunology*, **169**(11), 6343–6351.
[35] Turner, J., Gonzalez-Juarrero, M., Saunders, B.M. *et al*. (2001) Immunological basis for reactivation of tuberculosis in mice. *Infection and Immunity*, **69**(5), 3264–3270.
[36] Rook, G.A.W., Lowrie, D.B. and Hernandez-Pando, R. (2007) Immunotherapeutics for tuberculosis in experimental animals: Is there a common pathway activated by effective protocols? *Journal of Infectious Diseases*, **196**(2), 191–198.
[37] Iannaccone, M., Dorhoi, A. and Kaufmann, S.H. (2014) Host-directed therapy of tuberculosis: what is in it for microRNA? *Expert Opinion on Therapeutic Targets*, **18**(5), 491–494.
[38] Hawn, T.R., Matheson, A.I., Maley, S.N. and Vandal, O. (2013) Host-directed therapeutics for tuberculosis: can we harness the host? *Microbiology and Molecular Biology Reviews: MMBR*, **77**(4), 608–627.
[39] Gonzalez-Juarrero, M. (2012) Immunity to TB and targets for immunotherapy. *Immunotherapy*, **4**(2), 187–199.
[40] Johnson, B., Bekker, L.G., Ress, S. and Kaplan, G. (1998) Recombinant interleukin 2 adjunctive therapy in multidrug-resistant tuberculosis. *Novartis Foundation Symposium*, **217**, 99–106; 106–111.
[41] Johnson, B.J., Ress, S.R., Willcox, P. *et al*. (1995) Clinical and immune responses of tuberculosis patients treated with low-dose IL-2 and multidrug therapy. *Cytokines and Molecular Therapy*, **1**(3), 185–196.
[42] Suarez-Mendez, R., Garcia-Garcia, I., Fernandez-Olivera, N. *et al.* (2004) Adjuvant interferon gamma in patients with drug-resistant pulmonary tuberculosis: a pilot study. *BMC Infectious Diseases*, **4**, 44–52.
[43] Condos, R., Hull, F.P., Schluger, N.W. *et al*. (2004) Regional deposition of aerosolized interferon-gamma in pulmonary tuberculosis. *Chest*, **125**(6), 2146–2155.
[44] Condos, R., Rom, W.N. and Schluger, N.W. (1997) Treatment of multidrug-resistant pulmonary tuberculosis with interferon-gamma via aerosol. *Lancet*, **349**(9064), 1513–1515.
[45] Gao, X.F., Yang, Z.W. and Li, J. (2011) Adjunctive therapy with interferon-gamma for the treatment of pulmonary tuberculosis: a systematic review. *International Journal of Infectious Diseases*, **15**(9), e594–e600.
[46] Giosue, S., Casarini, M., Alemanno, L. *et al*. (1998) Effects of aerosolized interferon-alpha in patients with pulmonary tuberculosis. *American Journal of Respiratory and Critical Care Medicine*, **158**(4), 1156–1162.
[47] Tramontana, J.M., Utaipat, U., Molloy, A. *et al*. (1995) Thalidomide treatment reduces tumor necrosis factor alpha production and enhances weight gain in patients with pulmonary tuberculosis. *Molecular Medicine*, **1**(4), 384–397.
[48] Koo, M.S., Manca, C., Yang, G. *et al.* (2011) Phosphodiesterase 4 inhibition reduces innate immunity and improves isoniazid clearance of *Mycobacterium tuberculosis* in the lungs of infected mice. *PLoS One*, **6**(2), e17091.
[49] Subbian, S., Tsenova, L., O'Brien, P. *et al.* (2011) Phosphodiesterase-4 inhibition alters gene expression and improves isoniazid-mediated clearance of Mycobacterium tuberculosis in rabbit lungs. *PLoS Pathogens*, **7**(9), e1002262.

[50] Subbian, S., Tsenova, L., O'Brien, P. *et al*. (2011) Phosphodiesterase-4 inhibition combined with isoniazid treatment of rabbits with pulmonary tuberculosis reduces macrophage activation and lung pathology. *American Journal of Pathology*, **179**(1), 289–301.
[51] Wallis, R.S. (2007) Reactivation of latent tuberculosis by TNF blockade: the role of interferon gamma. *Journal of Investigative Dermatology Symposium Proceedings*, **12**(1), 16–21.
[52] Churchyard, G.J., Kaplan, G., Fallows, D. *et al*. (2009) Advances in immunotherapy for tuberculosis treatment. *Clinics in Chest Medicine*, **30**(4), 769–782.
[53] Ameglio, F., Casarini, M., Capoluongo, E. *et al*. (2005) Post-treatment changes of six cytokines in active pulmonary tuberculosis: differences between patients with stable or increased fibrosis. *International Journal of Tuberculosis and Lung Disease*, **9**(1), 98–104.
[54] Eum, S.Y., Lee, Y.J., Min, J.H. *et al*. (2010) Association of antigen-stimulated release of tumor necrosis factor-alpha in whole blood with response to chemotherapy in patients with pulmonary multidrug-resistant tuberculosis. *Respiration*, **80**(4), 275–284.
[55] Hernandez-Pando, R., Orozco, H., Arriaga, K. *et al*. (1997) Analysis of the local kinetics and localization of interleukin-1 alpha, tumour necrosis factor-alpha and transforming growth factor-β, during the course of experimental pulmonary tuberculosis. *Immunology*, **90**(4), 607–617.
[56] Hernandez-Pando, R., Orozco-Esteves, H., Maldonado, H.A. *et al*. (2006) A combination of a transforming growth factor-beta antagonist and an inhibitor of cyclooxygenase is an effective treatment for murine pulmonary tuberculosis. *Clinical and Experimental Immunology*, **144**(2), 264–272.
[57] Beamer, G.L., Flaherty, D.K., Assogba, B.D. *et al.* (2008) Interleukin-10 promotes *Mycobacterium tuberculosis* disease progression in CBA/J mice. *Journal of Immunology*, **181**(8), 5545–5550.
[58] Rosas-Taraco, A.G., Higgins, D.M., Sanchez-Campillo, J. *et al*. (2011) Local pulmonary immunotherapy with siRNA targeting TGFβ1 enhances antimicrobial capacity in *Mycobacterium tuberculosis* infected mice. *Tuberculosis*, **91**(1), 98–106.
[59] Keane, J. (2005) TNF-blocking agents and tuberculosis: new drugs illuminate an old topic. *Rheumatology*, **44**(6), 714–720.
[60] Keane, J., Gershon, S., Wise, R.P. *et al*. (2001) Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *New England Journal of Medicine*, **345**(15), 1098–1104.
[61] Ordway, D., Higgins, D.M., Sanchez-Campillo, J. *et al*. (2007) XCL1 (lymphotactin) chemokine produced by activated CD8 T cells during the chronic stage of infection with *Mycobacterium tuberculosis* negatively affects production of IFN-gamma by CD4 T cells and participates in granuloma stability. *Journal of Leukocyte Biology*, **82**(5), 1221–1229.
[62] Gonzalez-Juarrero, M., Woolhiser, L.K., Brooks, E. *et al*. (2012) Mouse model for efficacy testing of antituberculosis agents via intrapulmonary delivery. *Antimicrobial Agents and Chemotherapy*, **56**(7), 3957–3959.
[63] Lee, R.E., Hurdle, J.G., Liu, J. *et al*. (2014) Spectinamides: a new class of semisynthetic antituberculosis agents that overcome native drug efflux. *Nature Medicine*, **20**(2), 152–158.
[64] Condos, R., Raju, B., Canova, A. *et al*. (2003) Recombinant gamma interferon stimulates signal transduction and gene expression in alveolar macrophages in vitro and in tuberculosis patients. *Infection and Immunity*, **71**(4), 2058–2064.
[65] Giosue, S., Casarini, M., Ameglio, F. *et al*. (2000) Aerosolized interferon-alpha treatment in patients with multi-drug-resistant pulmonary tuberculosis. *European Cytokine Network*, **11**(1), 99–104.
[66] Aerosol Vaccines for Tuberculosis Workshop Summary G. (2015) Developing aerosol vaccines for *Mycobacterium tuberculosis*. Workshop Proceedings, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA, April 9, 2014. *Vaccine*, **33**(26) 3038–3046.
[67] Rosenthal, S.R., McEnery, J.T. and Raisys, N. (1968) Aerogenic BCG vaccination against tuberculosis in animal and human subjects. *Journal of Asthma Research*, **5**(4), 309–323.
[68] Bivas-Benita, M., Lin, M.Y., Bal, S.M. *et al*. (2009) Pulmonary delivery of DNA encoding *Mycobacterium tuberculosis* latency antigen Rv1733c associated to PLGA-PEI nanoparticles enhances T cell responses in a DNA prime/protein boost vaccination regimen in mice. *Vaccine*, **27**(30), 4010–4017.

[69] Fourie, P.B., Germishuizen, W.A., Wong, Y.L. and Edwards, D.A. (2008) Spray drying TB vaccines for pulmonary administration. *Expert Opinion on Biological Therapy*, **8**(7), 857–863.
[70] Garcia-Contreras, L., Fiegel, J., Telko, M.J. *et al*. (2007) Inhaled large porous particles of capreomycin for treatment of tuberculosis in a guinea pig model. *Antimicrobial Agents and Chemotherapy*, **51**(8), 2830–2836.
[71] Garcia-Contreras, L., Sung, J.C., Muttil, P. *et al*. (2010) Dry powder PA-824 aerosols for treatment of tuberculosis in guinea pigs. *Antimicrobial Agents and Chemotherapy*, **54**(4), 1436–1442.
[72] Jin, T.H., Tsao, E., Goudsmit, J. *et al*. (2010) Stabilizing formulations for inhalable powders of an adenovirus 35-vectored tuberculosis (TB) vaccine (AERAS-402). *Vaccine*, **28**(27), 4369–4375.
[73] Garcia-Contreras, L., Wong, Y.L., Muttil, P. *et al*. (2008) Immunization by a bacterial aerosol. *Proceedings of the National Academy of Sciences of the United States of America*, **105**(12), 4656–4660.
[74] Feng, G., Jiang, Q., Xia, M. *et al*. (2013) Enhanced immune response and protective effects of nano-chitosan-based DNA vaccine encoding T cell epitopes of ESAT-6 and FL against *Mycobacterium tuberculosis* infection. *PLoS One*, **8**(4), e61135.
[75] Darrah, P.A., Bolton, D.L., Lackner, A.A. *et al*. (2014) Aerosol vaccination with AERAS-402 elicits robust cellular immune responses in the lungs of rhesus macaques but fails to protect against high-dose *Mycobacterium tuberculosis* challenge. *Journal of Immunology*, **193**(4), 1799–1811.
[76] White, A.D., Sibley, L., Dennis, M.J. *et al*. (2013) Evaluation of the safety and immunogenicity of a candidate tuberculosis vaccine, MVA85A, delivered by aerosol to the lungs of macaques. *Clinical and Vaccine Immunology*, **20**(5), 663–672.
[77] Rosas-Taraco, A.G., Higgins, D.M., Sanchez-Campillo, J. *et al*. (2009) Intrapulmonary delivery of XCL1-targeting small interfering RNA in mice chronically infected with *Mycobacterium tuberculosis*. *American Journal of Respiratory Cell and Molecular Biology*, **41**(2), 136–145.
[78] Higgins, D.M., Sanchez-Campillo, J., Rosas-Taraco, A.G. *et al*. (2008) Relative levels of M-CSF and GM-CSF influence the specific generation of macrophage populations during infection with *Mycobacterium tuberculosis*. *Journal of Immunology*, **180**(7), 4892–4900.
[79] Kaur, J., Muttil, P., Verma, R.K. *et al.* (2008) A hand-held apparatus for "nose-only" exposure of mice to inhalable microparticles as a dry powder inhalation targeting lung and airway macrophages. *European Journal of Pharmaceutical Sciences*, **34**(1), 56–65.
[80] Garcia-Contreras, L., Muttil, P., Fallon, J.K. *et al*. (2012) Pharmacokinetics of sequential doses of capreomycin powder for inhalation in guinea pigs. *Antimicrobial Agents and Chemotherapy*, **56**(5), 2612–2618.
[81] Higgins, D.M., Sanchez-Campillo, J., Rosas-Taraco, A.G. *et al*. (2009) Lack of IL-10 alters inflammatory and immune responses during pulmonary *Mycobacterium tuberculosis* infection. *Tuberculosis*, **89**(2), 149–157.
[82] Redford, P.S., Murray, P.J. and O'Garra, A. (2011) The role of IL-10 in immune regulation during *M*. t*uberculosis* infection. *Mucosal Immunology*, **4**(3), 261–270.

Section 5

Future Opportunities

17

Treatments for Mycobacterial Persistence and Biofilm Growth

David L. Hava and Jean C. Sung*

17.1 Introduction

The failure of the pharmaceutical industry to address the emergent problem of antibiotic resistance through the development of new antibacterial drugs is well documented and has become an increasingly crucial public health problem in the last quarter century [1]. Nowhere has this been more evident than in treatments for *Mycobacterium* spp., and in particularly *M. tuberculosis*, where the primary first- and second-line drugs used in treatment regimens were all discovered before 1980 [2]. In the intervening time, increasingly drug-resistant strains have emerged including those classified as drug-resistant, multi-drug-resistant (MDR-TB), and extensively-drug-resistant (XDR-TB), the latter of which are resistant to at least 4 different classes of antibiotics [3]. There is an urgent need for new therapeutic alternatives for both *M. tuberculosis* and non-tuberculoid mycobacteria (NTM) infections. This need has led to an influx of new drug candidates and a large number of ongoing clinical trials evaluating both repurposed antibiotics used in other infections and drugs with new targets, the majority of which are bactericidal antibiotics aimed at killing growing bacteria [2].

Antibiotic treatment regimens for *M. tuberculosis* vary in length depending on the drug susceptibility of the infecting strain. For drug-susceptible strains, a 6-month course of antibiotics is given that includes an intensive 2-month phase of 4 antibiotics followed by a 4-month continuation phase of 2 antibiotics [4]. In cases of MDR-TB, treatment regimens

* Corresponding author, *Pulmatrix Inc., 99 Hayden Ave, Suite 390, Lexington, MA 02421, USA*

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

are of questionable benefit and may extend for as long as 24 months [5]. Treatment regimens for other pulmonary mycobacterial infections such as the NTM species *M. avium* complex (MAC) and *M. abscessus* are equally intensive and may include a combination of systemically administered drugs together with drugs inhaled directly into the lungs [6]. In all instances, the treatment burden of these drug regimens is high and long-term adherence difficult. The necessity of long treatment regimens with multiple antibiotics highlights the ability of *Mycobacterium* spp. to persist long term and tolerate antibiotic exposure through a variety of mechanisms that include the emergence of drug-resistant strains, drug-tolerant persisters and specialized growth in biofilms. New strategies in addition to traditional antibiotics may be useful in potentiating drug activity and increasing the likelihood of eradicating these difficult-to-treat infections.

17.2 Mycobacterial Persistence and Drug Tolerance

Prolonged exposure to antibiotics results in the selection of antibiotic-resistant strains that can survive treatment and outcompete susceptible strains in the presence of drug. Mycobacterial drug-resistance mechanisms have emerged that parallel those found in other bacterial species [1]. In addition to genetic mechanisms of bacterial drug resistance, bacteria have evolved adaptations that allow them to chronically infect mammalian hosts and persist in the face of both chemotherapy and host-responses to infection. Bacterial persistence allows small, sub-populations of bacteria to survive in the presence of bactericidal activity and then grow when the drug is removed [7]. Persistence is an actively maintained state that involves both spontaneous changes in gene expression and orchestrated responses to extracellular cues to ultimately reduce growth rate and avoid bactericidal killing [7]. Mycobacterial persistence results in antibiotic tolerance, which can be seen in both the difficulty of sterilizing infection and the high rates of infection relapse. In the presence of isoniazid (INH), *M. smegmatis* persists through a balance of cell division and death based on the stochastic expression of KatG, a catalase-peroxidase that activates INH and leads to bacterial death in high-KatG-expressing cells [8], with random bacteria expressing low levels of KatG exhibiting greater INH tolerance. Likewise, mycobacteria divide in an asymmetric manner that results in heterogeneous cultures with cells that vary in elongation rate, size and sensitivity to different antibiotic classes [9], and stressed growth conditions reduce mycobacterial metabolic rates and growth, resulting in tolerance to a broad scope of antibiotics [10]. These relatively random processes resulting in antibiotic tolerance result in a meaningful fraction of the bacterial population surviving each round of antibiotic exposure and driving persistence.

While stochastic processes lead to the selection of antibiotic-tolerant sub-populations, it is clear that bacteria also respond to environmental cues to induce states of antibiotic tolerance and persistence. Mycobacteria are able to maintain long-term viability in low-nutrient or starvation conditions that resist killing by INH and rifampicin (RIF) in a state that requires induction of the stringent response and RelA [11]. Similar persistence responses exist in other bacteria in response to DNA damage, oxidative stress and quorum sensing [7]. Interestingly, induced mechanisms of persistence are similar to mechanisms that are traditionally thought of as drug-resistant strategies. One example of this is the induced expression of antibiotic-efflux pumps in mycobacteria during macrophage infection,

which supports the emergence of antibiotic tolerance during intracellular growth [12]. In a number of other bacteria, the induced expression of nitric oxide and hydrogen sulfide provides a protective effect against antibiotics and protection against oxidative stress [13, 14]. Mechanistically, mycobacteria achieve a persistence state through the combined effects of changes in growth rate and active mechanisms, which might lead to the achieved balance of both active division and cell death [8].

A common mechanism utilized by bacteria to persist in harsh environments both *in vivo* and in the environment is the formation of multicellular biofilm communities. Biofilms form as cooperative communities on both biological and artificial surfaces to provide protective advantages to the community that are not otherwise achieved as planktonically growing bacteria [15]. In many bacteria, the same environmental cues that induce persistence also drive biofilm formation and maturation, and lead to antibiotic tolerance. These include bacterial cell-to-cell signaling, induction of the stringent response resulting from nutrient limitation or starvation, and increasing anti-oxidant defenses [16–19]. To form biofilms, bacteria differentiate from motile, planktonic bacteria to multi-bacterial chains or aggregates that produce and secrete a matrix of extracellular polysaccharide (EPS) that surrounds and imbeds the bacterial community in a three-dimensional structure. Mature biofilms afford protection as a barrier to antibiotic penetration and create a nutrient environment where antibiotic tolerance can be maintained. *Pseudomonas aeruginosa*, an opportunistic pathogen that chronically colonizes the lungs of patients with Cystic Fibrosis (CF), forms biofilms on lung epithelial cell surfaces where innate defenses of the airway such as the mucus barrier and mucociliary clearance are diminished. Mature *P. aeruginosa* biofilms are entrenched in a thick polysaccharide layer that provides a barrier to antibiotics and creates a hypoxic, low-oxygen environment where bacterial growth may be anaerobic and nutrients are limited [20, 21]. Environmental changes that alter bacterial growth and gene-expression patterns and trigger persistence and biofilm formation are also at work in biofilms where signals are generated that cause the biofilm to disassemble or disperse. As more detailed understandings of these signaling pathways emerge the perturbation or utilization of these signals to manipulate antibiotic sensitivity should be explored as a therapeutic strategy to either dissipate biofilms or to increase their sensitivity to antibiotic treatment.

17.3 Mycobacterial Multicellular Growth

Classically, *Mycobacterium* spp. are intracellular pathogens that have adapted strategies to survive within host macrophages through the alteration of phagolysosome maturation or by phagosomal escape resulting in evasion of host responses and bacterial killing [22, 23]. For NTM species, biofilms form in environmental sources and are important for bacterial growth and survival in these systems, owing in part to the ability of the biofilm to support growth in low-nutrient environments where they are exposed to antimicrobial treatments [24, 25]. Importantly, these environmental biofilms are enriched in areas that may be sources of human exposure to aerosolized bacteria [26, 27], suggesting that treatments to prevent or dissipate biofilms could be considered beyond therapies for humans.

Whether long-term persistence of *Mycobacterium* spp. involves the formation of biofilms in humans is an open question and difficult to study; however, pathological evidence of multicellular growth of *M. tuberculosis* and *M. abscessus* has been described [28–30].

Recently, proteins that are uniquely expressed during mycobacterial biofilm growth have been identified and subsequently detected with sera collected from infected guinea pigs [31]. While the functions of many of these proteins are not known, the data suggest that they are expressed *in vivo* and that structures similar to those cultured *in vitro* may form in animals. Further genetic analysis of the role of these proteins in both biofilm formation and infection may further establish a link between biofilm culture *in vitro* and its role *in vivo*.

Despite the limited clinical evidence that *Mycobacterium* spp. form biofilms during infection, it is clear that *Mycobacterium* spp. are able to grow multicellularly in structures that are similar to classical biofilms *in vitro*. These include multicellular growth in cords, which is associated with virulence and requires trehalose dimycolate (TDM) [32] and growth in pellicles at the air–liquid interface, which is a multicellular film of bacteria that spread across the culture surface and is common to many strains of *M. tuberculosis* isolated worldwide [32–34]. Cording and growth in pellicles are distinct phenotypes that can be genetically separated [35], with pellicles generally associated with a biofilm form of growth. Like classical biofilms formed by Gram-negative species such as *P. aeruginosa, M. tuberculosis* and NTM biofilms are tolerant to antibiotic treatment *in vitro* [36, 37]. When cultured as planktonic bacteria over a 5-day period, INH treatment results in a greater than 4 $\log_{10}$ reduction in *M. tuberculosis* viability compared with a 1-to-2 $\log_{10}$ reduction during biofilm culture. Similar results were seen following RIF treatment; however, the overall sensitivity of the biofilms to RIF treatment was greater than that for INH [36]. The protection afforded by biofilm formation against antibiotic treatment is further evident by the ability of a biofilm to confer protection to drug-sensitive bacteria that are presumably encased in the larger bacterial structure [35]. Collectively, *in vitro* data suggest that the growth of *Mycobacterium* spp. in biofilms may account for some of the difficulty in treating infections through improved drug tolerance *in vivo*.

17.4 Mycobacterial Lipids Involved in Biofilm Formation

When grown in biofilms, Gram-positive and Gram-negative bacteria secrete large amounts of EPS that surround the bacteria and modulate many of the properties of the biofilm community including stability, barrier function and adhesion. Mycobacterial genomes do not contain genes for EPS production [38], but replicate the function of EPS through the secretion of unique lipids similar to those that make up the mycobacterial cell wall. Cell wall lipids and their biosynthetic pathways therefore have a critical role in biofilm attachment, maturation and in the resistance to antibiotics (Table 17.1).

Rapidly growing strains of *M. smegmatis* and *M. abscessus*, and slow growing members of the *M. avium* complex (MAC), produce glycopeptidolipids (GPLs) on their outermost cell surface [50]. In *M. smegmatis*, mutants unable to produce GPLs are defective in sliding motility and biofilm formation [39, 40]. Among the mutants identified were those with transposon insertions in the *mps* genes, which are involved in the formation of the lipopeptide core of GPLs, a mutation in *mmpL4b* (*tmtpC*) that blocks GPL transport, and a third mutation that results in non-acetylated GPLs [39, 40, 51]. In *M. abscessus,* GPL expression and transport also depend on MmpL4b, and deletion of *mmpL4* results in loss of biofilm formation, but interestingly a gain in the ability to replicate within monocyte-derived macrophages [41]. The *mps* (*pstAB*) gene has a similar role in *M. avium* in catalyzing the

Table 17.1 *Mycobacterial lipids implicated in biofilm growth*

Lipid	Species	Key data	Gene/Protein function	References
Glycopeptidolipids (GPLs)	*M. smegmatis*	Mutation in *mps*	GPL core biosynthesis	[39]
		Mutation in *mmpL4b*	GPL transport	[40]
		Mutation in *atf1*	GPL acetylation	[40]
	M. abscessus	Mutation in *mmpL4b*	GPL transport	[41]
	M. avium	Mutation in *mps*	GPL core biosynthesis	[42, 43]
Free C_{56}–C_{68} methoxymycolates	*M. smegmatis; M. tuberculosis*	Mutation in *groEL1*	Chaperone required for mycolic acid biosynthesis	[44, 45]
		Mutation in Msmeg_1529	Enzymatic release of mycolic acids	[46]
Ketomycolic acids	*M. tuberculosis*	Mutation in *mmaA4*	Methyltransferase	[35]
Trehalose dimycolate (TDM)	*M. smegmatis; M. tuberculosis*	Mutation in *fbpA*	Organization of TDM in cell wall	[46]
		Inhibitors of MmpL3	Trehalose monomycolate (TMM) transport	[47]
Monomerylmycol diacylcglycerol	*M. smegmatis*	Mutation in *mmpL11*	Lipid transport	[48]
Mycolate ester wax	*M. smegmatis*	Mutation in *mmpL11*	Lipid transport	[48]
Arabinan biosynthesis	*M. smegmatis; M. tuberculosis*	Inhibitors of DprE1	Arabinan biosynthesis	[49]

synthesis of the GPL core and mutants are deficient at colonizing surfaces and forming biofilms [42, 43]. Because GPLs are localized to the outermost surface of the cell wall, a model was proposed in which GPLs modify the bacterial cell surface to create a hydrophobic surface that drives *M. smegmatis* bacterial attachment and the early stages of biofilm formation [39].

Lipid components of the mycobacterial cell wall are also important for the structure and architecture of biofilms. In particular, mycolic acid synthesis and secretion have a significant role. Mycolic acid biosynthesis and mycolic acid lipid profiles differ during planktonic and biofilm growth, with increased synthesis of shorter C_{56}–C_{68} mycolic acids and a higher proportion of free mycolic acids during biofilm growth, which are predominantly methoxymycolates [35, 36, 44]. The release of free mycolic acids from the cell surface occurs through the enzymatic cleavage of newly synthesized trehalose dimycolate (TDM), which occurs differentially during stages of biofilm formation and maturation [46]. The accumulation of free or surface-bound methoxymycolates during biofilm growth may provide an analogous function to EPS secretion in stabilization and protection against external insults.

Genetic analysis of the mycolic acid biosynthetic pathway further supports the role of these lipids in multiple stages of biofilm development. Biofilm formation and mycolic acid

synthesis both require the non-essential chaperone GroEL1. The role for GroEL1 is specific to biofilm growth and requires physical interaction with the type II Fatty Acid Synthase (FAS) complex through association with KasA [44]. The type II FAS system is involved in fatty acid elongation and KasA catalyzes the acyl extension of mycolic acids. Transcriptional down regulation of these genes results in alterations in lipid composition and impaired biofilm formation [45]. Likewise, the ability to synthesize ketomycolic acids is essential for the initiation of biofilm growth and dependent on the expression of the methyltransferase MmaA4 [35], a protein that is also required for virulence [52] and that is expressed during human infection [53].

Downstream of mycolic acid biosynthesis, steps in both the transport of mycolic acids and the final stages of mycolic acid localization in the cell wall have roles in biofilm growth. Bacteria with mutations in gene encoding the Antigen 85 complex, which organizes TDM in the cell envelope [54], are defective at biofilm formation, with the strongest defect observed in a Δ*fbpA* (Ag85A) strain [46]. Consistent with their role in biofilm development, Ag85A, GroEL1 and others involved in cell-wall biosynthesis are expressed during biofilm formation and specifically coincide at time points where marked changes in morphology of the biofilm occur [55]. Understanding the transport of long-chain mycolic acids across the mycobacterial cell membrane has been an area of intensive focus and study [56]. Recently, MmpL3, a large transmembrane protein, was identified as the transporter of trehalose monomycolate (TMM), the precursor to TDM [47]. Inhibitors of MmpL3 have been identified that are bactericidal against aerobically growing bacteria, but not against nonreplicating anaerobic growth [47]; however, a direct effect of these drugs on biofilm growth has not been tested. The *mmpL3* and *mmpL11* genes are the only two MmpL-type proteins expressed by *M. tuberculosis* that are conserved across all *Mycobacterium* spp. [57]. In *M. smegmatis*, MmpL11 is required for transport of monomeromycolyl diacylglycerol and mycolate ester wax across the plasma membrane, and mutants in *mmpL11* have altered cell-wall composition and reduced ability to form mature biofilms [48]. Whether the alteration of the lipid composition in the *mmpL11* has indirect effects on biofilm formation through changes in the bacterial cell wall or if its lipid substrates play a specific role in stages of biofilm formation and/or maturation remains to be determined.

17.5 Therapies to Treat Mycobacterial Biofilms and Persistence

17.5.1 Therapies to Treat Mycobacterial Biofilms

The elucidation of pathways important for biofilm formation in different *Mycobacterium* spp. has resulted in a number of possible drug targets for disrupting biofilm growth (Figure 17.1). Interestingly, many of the same pathways identified as critical to biofilm formation have been cultivated as more general targets of antimycobacterial drug discovery and development. Therefore, several drugs in development may provide the benefit of bactericidal activity against both planktonic bacteria and multicellular bacterial growth. One such target is MmpL3, the cell-membrane protein required for TMM translocation. With the identification of MmpL3 as the TMM transporter, a small molecule (AU1235) inhibitor of TMM transport was identified with bactericidal activity against *M. tuberculosis* [47]. Following the identification of MmpL3, two additional small-molecule inhibitors of

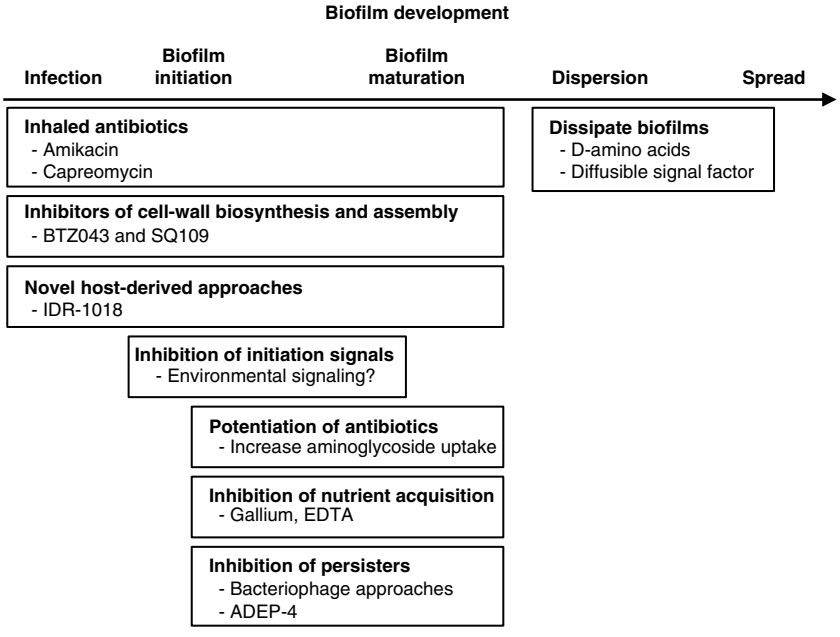


Figure 17.1 *Stages of biofilm development as potential therapeutic targets. General stages of biofilm growth are shown as infection, initiation, maturation, dispersal and spread. Therapeutic strategies and specific examples discussed in the text are shown in each box below. The location of each box on the developmental timeline suggests where each therapy might be most applicable as an interventional therapy based on the current understanding of mycobacterial biofilms and existing data for each strategy*

cell-wall assembly, SQ109 and BM212, were found to inhibit the same target [58, 59]. SQ109 has potent bactericidal activity against *M. tuberculosis* cultures *in vitro* and acts synergistically with INH and RIF [58, 60]. While MmpL3 has not been directly implicated in biofilm development, the important role that mycolic acids have in the process would suggest that, in addition to potent effects on planktonic growth, SQ109 may also be effective at killing bacteria in biofilms. SQ109 has completed Phase 2 clinical testing and is the focus of ongoing clinical development (see https://clinicaltrials.gov/ct2/show/NCT01218217 - accessed 23rd May 2016).

In addition to novel bactericidal antibiotics, the development of new approaches to specifically treat multicellular mycobacterial growth may be advantageous to incorporate into existing therapeutic regimens to be used in combination with antibiotics (Figure 17.1). Such compounds may be developed to specifically target biofilms or more generally target bacterial persisters and drug-tolerant bacteria. To this end, a significant challenge exists in designing growth conditions and selective strategies to not only identify genes that are important for growth, but are required for survival in a slower growing persistence state; however, strategies to overcome these hurdles are emerging [49, 61, 62]. Using a high-throughput screen and *M. smegmatis* biofilm growth, Wang *et al*. identified two classes of compounds that either inhibit mycobacteria growth in biofilm conditions or prevented

biofilm formation [49]. The lead compound identified (TCA1) exhibited similar *in vitro* bactericidal activity to RIF and INH against actively growing cultures, but with the advantage of preventing the outgrowth of resistant strains observed for the latter drugs. Further, TCA1 exhibited superior killing against nonreplicating *M. tuberculosis* compared with RIF and comparable activity in mouse models of infection to both RIF and INH [49]. TCA1 targets DprE1, a component of an essential enzyme involved in cell-wall arabinan biosynthesis [63]. Coincidentally, DrpE1 is the target of benzothiazinones, a new class of antimycobacterial drugs with potent activity exhibiting activity at nanomolar concentrations *in vitro* [64] and the focus of intensive drug-development efforts. The most advanced benzothiazinone compound, BTZ043, is currently in preclinical development [2]. Collectively, the data suggest that cell-wall arabinan biosynthesis furthers the role lipids have in mycobacterial biofilm formation and growth and that through the targeting of DrpE1 multiple stages of mycobacterial growth may be impacted.

17.5.2 Therapies to Disrupt Nutrient Acquisition and Persistence

Mycobacterium spp. are similar to other bacteria in that they respond to changes in nutrient conditions or external stimuli to persist and survive. One response to environmental or nutrient stress is to induce cellular processes to enable persistence, alter nutrient needs and possibly form biofilms [7, 33]. This is evident by the dependence of *in vitro* biofilm formation on specific growth conditions with specific nutrient requirements such as iron (Fe) or zinc [36, 65]. Iron has a significant role in the formation and development of *P. aeruginosa* biofilms and iron acquisition has been targeted as an anti-biofilm approach [66–69]. Given the similar role of Fe in the formation of mycobacterial biofilms, therapeutic approaches aimed at Fe acquisition and metabolism may be similarly applicable.

Approaches focused on the disruption of bacterial metal cation and Fe acquisition attempt to mimic the effect host Fe-scavenging proteins have on biofilm development [66]. Gallium (Ga) is a semi-metallic element with antibacterial activity against a number of Gram-positive and Gram-negative bacteria through the disruption of Fe uptake and the function of bacterial Fe-binding proteins [70]. A principle mechanism by which this occurs is through the displacement of Fe on bacterial siderophores, leading to Ga uptake specifically by bacterial cells [71]. *In vitro*, Ga blocks iron uptake by *P. aeruginosa* in a dose-dependent manner, prevents the establishment of biofilms and kills bacteria growing in biofilms, an attribute lacking in antibiotics [72, 73]. The safety and efficacy of intravenous (IV) Ga in CF has been advanced to clinical testing [74].

A second approach to disrupting biofilms may be to directly chelate extracellular metal cations required for biofilm maintenance and maturation.One example of this approach is to utilize Fe chelation in combination with existing antibiotics. Growth of *P. aeruginosa* biofilms on CF-derived epithelial cells requires Fe and can be prevented through treatment with Food and Drug Administration (FDA)-approved iron chelators [75]. More interestingly, when used in combination with tobramycin, Fe chelators disrupt biofilms through the chelation of Fe and the antibiotic treatment significantly reduce bacterial viability [75]. Similar results have been obtained using the broad metal chelator ethylenediaminetetraacetic acid (EDTA) in combination with antibiotics to disrupt biofilms formed by *Staphylococcus* spp. [76] and by *P. aeruginosa* [77], an effect in the latter case that was dependent on the chelation of Fe and calcium. The important role of siderophore-mediated iron uptake

during mycobacterial biofilm growth [65] suggests that strategies such as Fe replacement with Ga or chelation may be similarly effective strategies (Figure 17.1).

In addition to the perturbation of biofilm growth, the manipulation of local metabolite concentrations may allow the sensitization of mycobacteria to antibiotics, whether grown in a biofilm or in a persistent state. In *E. coli,* the addition of carbon sources utilized in glycolysis to antibiotic treatment of persister cultures potentiates the activity of aminoglycosides [78]. This effect is specific to aminoglycosides and a result of increasing proton-motive force (PMF) and aminoglycoside uptake, without reverting persisters to a normal growth state. The combined effect of metabolites and aminoglycosides was also evident in treating biofilms both *in vitro* and *in vivo* [78]. Similar results were found using *Staphylococcus aureus*, although, interestingly, fructose rather than mannitol and glucose exerted the greatest potentiation [78]. Peng and colleagues extended these findings using the amino acid alanine to demonstrate that, by similarly inducing PMF and aminoglycoside uptake, sufficient concentrations of intracellular drug could be achieved to kill antibiotic-resistant bacteria grown planktonically and in biofilms [79]. The simplicity of the metabolites that potentiate the effects of antibiotics may allow them to be easily incorporated into drug formulations delivered to infection sites where the combined activity may be observed.

The redundancy of pathways that may lead to persistence poses a significant challenge in the development of a single therapeutic to inhibit specific bacterial signaling pathways [1]. As an alternative approach, compounds that activate cellular processes to broadly affect bacterial physiology have been identified that kill both persister and actively growing cultures [80]. ADEP-4, the compound identified, activates ClpP, a protease that normally selectively recognizes and degrades misfolded proteins. Through the activation of ClpP with ADEP-4, ClpP becomes a non-selective protease that kills *S. aureus* persisters in both broth culture and biofilms and eradicates difficult-to-treat infections in mice when used in combination with antibiotics [80]. Additional strategies have been described that interfere with stress responses that may converge on common pathways of persistence, including interference of bacterial SOS response, oxidative stress response or stringent response [7]. Several of these approaches utilize bacteriophages to exogenously express proteins that interfere with these responses, including uncleavable forms of the LexA repressor or overexpression of the SoxR transcriptional regulator to enhance antibiotic therapy [81]. Similar bacteriophage strategies have been proposed to sensitize biofilms to antibiotics or to disperse biofilms using species-targeted bacteriophages expressing enzymes that will degrade the protective extracellular polysaccharide surrounding the biofilm [82]. The use of bacteriophages for the treatment and prevention of mycobacterial infections has been suggested [83] and the use of mycobacteriophages in mycobacterial genetics offers tools that may be adapted to use similar approaches to genetically modify *Mycobacterium* spp. during infection.

17.5.3 Treatments for Biofilm Dispersion

The study of mycobacterial biofilms has primarily focused on characterization of growth conditions and genetic requirements for biofilm formation, but not the specific signals that trigger formation or disassembly of such structures. While killing bacteria in biofilms may be achievable, the dissipation of biofilms in combination of antibiotic treatment may provide an alternative method to eradication. For this approach, care must be taken to assure that

biofilm dissipation does not result in a significant immune response resulting from releasing a large number of bacteria from these structures simultaneously. In other species, signaling systems exist that enable individual bacteria to sense their environment and bacterial cell density to trigger stages of biofilm formation and dissipation. These signaling systems include quorum sensing, secreted signals causing biofilm disassembly and secreted bacterial enzymes that interfere with competing bacteria [84–86]. An attractive approach to modulating biofilm dispersal or increasing drug sensitivity would be to build upon the understanding of cross-species interference or biofilm-dispersal signals to identify therapeutic compounds to be used in mycobacterial infections (Figure 17.1).

In the specific case of mycobacterial biofilms a greater understanding of the signals that drive biofilm formation or dispersion will allow direct targeting of these processes for therapeutic purposes. Of concepts that have been identified in other species, many may not be suitable for mycobacterial biofilms as the distinct cell-wall structure and lipid-rich extracellular matrix of their biofilms distinguish them from those that form polysaccharide-based biofilm matrices. For example, *P. aeruginosa* and *S. aureus* produce D-amino acids during biofilm growth that lead to disassembly of the biofilm by their incorporation into cell-wall peptidoglycan, leading to release of proteins within the extracellular matrix. Likewise, treatment of biofilms with D-amino acids can dissipate preformed biofilms [87–89]. *M. tuberculosis* has a peptidoglycan layer that is essential for survival and is similar to other bacteria in both structure and genomic organization [90]. However, it is not obvious that D-amino acids would penetrate the hydrophobic lipid coat surrounding the bacterium to reach the peptidoglycan or if a similar effect on the lipid components of the biofilm would be observed.

Similar to the effect of D-amino acids, many bacteria respond to a secreted fatty acid signal similar to diffusible signal factor (DSF) originally identified in *Xanthomonas campestris*, that disperses and prevents biofilm formation of a number of bacteria [86, 91]. The most potent compound identified, *cis*-2-decenoic acid [(*Z*)-dec-2-enoic acid] , is present in low nanomolar concentrations in growth media and is active at dispersing biofilms at concentrations of 10 nM [86]. In other studies, DSF treatment sensitizes *Bacillus cereus* to antibiotic treatment, inhibits biofilms and reduces persistence [92]. Similarly, DSF treatment of *M. smegmatis* cultures *in vitro* lowered minimum inhibitory concentration (MIC) values of several antibiotics when used in combination, suggesting that such compounds may enhance antibiotic potency during planktonic growth [92]. Whether similar factors could be used to break persistence or enhance the activity of antibiotics against mycobacterial biofilms remains to be tested.

17.5.4 Treatments Derived from Host Innate Defenses

A growing understanding of how innate host defenses may differentially affect stages of bacterial growth or how these defenses are inactivated during infection may allow for development of novel therapies derived from host peptides. The human cationic peptide LL-37 is an example of such a strategy, as LL-37 exhibits weak bactericidal activity against *P. aeruginosa*, but potently inhibits biofilm formation and manipulates bacterial gene expression to alter established biofilms [93]. The basic understanding of cationic peptide activity under different conditions led to the development and discovery of a synthetic peptide, IDR-1018 [94], which is an exciting example of this premise. IDR-1018 exhibits immunomodulatory activity, by both inducing chemokine secretion to help protect against

infection and attenuating pro-inflammatory cytokine responses [94], and has exhibited a protective effect in a number of preclinical bacterial infection models including *M. tuberculosis* [95]. Related to biofilm treatments, IDR-1018 prevents biofilm formation and eradicates mature biofilms formed by both Gram-negative and Gram-positive bacteria [96]. This activity is independent of direct bactericidal activity, but rather through the inhibition of bacterial stringent stress responses, which are broadly conserved across species including *Mycobacterium* spp. [96, 97]. Further, in humans with respiratory disease or a history of smoking, human neutrophil peptides (HNPs) become inactivated through the ADP ribosylation of arginine residues [98, 99]. Through the replacement of arginine with ornithine HNP-1, the cytotoxic and bactericidal activities of HNP-1 can be separated [100] and may provide the opportunity to develop more potent analogues that resist inactivation during infection or disease.

17.5.5 Treatments with Inhaled Antibiotics

While approaches for the eradication of established biofilms in chronic infections are only in early development, it is likely that these new approaches will be used in combination with antibiotics to maximize clinical efficacy. To target these therapies to the primary sites of infection, delivery of drugs directly to the lungs is an attractive approach and one that has been used clinically to treat *P. aeruginosa* infection in CF by using inhaled antibiotics. The mainstay of inhaled antibiotic therapy in CF since its approval in 1997 has been the use of the inhaled aminoglycoside tobramycin [101]. Inhaled tobramycin is currently available as tobramycin inhalation solution (TIS) for nebulization (TOBI®; Novartis AG) and a more recently approved dry-powder formulation [TOBI PodHaler® (Tobramycin Inhalation Powder; TIP); Novartis, AG]. Intermittent use of aerosolized TIS using monthly cycling of periods of on-off treatment significantly reduced sputum bacterial burden by 0.8 to 2.2 $\log_{10}$ colony-forming units (CFU) per gram of sputum over a period of 20 weeks and improved pulmonary function [10% increase in forced expiratory volume in 1 second (FEV1)] over the same time period compared with placebo [102]. TIP was developed using a novel dry-powder particle engineering technology, called Pulmospheres™, which allow for high drug loading and reduced treatment burden compared with nebulized liquid drugs with a similar treatment benefit in clinical trials [103]. The success of inhaled tobramycin has led to the development and approval of inhaled aztreonam (Gilead Sciences, Inc.) and colistimethate sodium (Forest Laboratories, Inc.) for treatment of *P. aeruginosa*, with additional non-pseudomonal therapies in development [104].

NTM infection in CF has gained increasing attention over the last decade. Rates of NTM infection in CF are increasing and are associated with increased lung-function decline [30, 105]. *M. abscessus* and MAC are the most common NTM infections in CF; however, the prevalence of different strains differs by geography [106]. Diagnosing pulmonary NTM infection is particularly difficult due to underlying disease and nonspecific symptomatology. Treatment regimens involve 12-month regimens of oral and IV antibiotics, including macrolides and aminoglycosides [107].

While appreciation of the negative effects of pulmonary NTM infection has grown, new antibiotic development focused on treating these infections has lagged. Inhalation of antibiotics directly to the lungs has the potential to dramatically improve treatment options by directly targeting the site of infection and achieving high local drug concentrations that

may improve bacterial killing and overcome low levels of drug resistance. Inhaled amikacin is in development as a liposomal formulation focused on treating pulmonary NTM infections [Liposomal Amikacin for Inhalation (LAI); Insmed, Inc.] [108]. The liposomal formulation provides several advantages to pulmonary drug delivery over the delivery of free, unencapsulated drug. Lung-delivered liposomal amikacin exhibits biphasic clearance from rat lungs, with a significant proportion of the drug exhibiting a lung half-life of more than 24 h and improved *in vivo* activity compared with free drug [109]. Additionally, the liposomal formulations penetrate bacterial biofilms and release amikacin over a long time period, a process that involves the activity of bacterial virulence factors [109]. The promising preclinical data using LAI led to clinical development for the treatment of *P. aeruginosa* and NTM infection in both CF and non-CF disease. In a Phase 2 randomized, placebo-controlled clinical trial of LAI in NTM infection, 90 subjects were randomized to LAI or placebo and treated once daily for a 12-week double-blind phase followed by a 12-week open-label phase where all subjects were administered LAI [110]. The majority of patients (81%) were non-CF and had MAC infection (64%). While the primary endpoint of change from baseline in the full semi-quantitative scale for mycobacterial culture was not statistically significant, a high percentage of patients achieved NTM culture conversion to negative. Eleven of 44 patients achieved culture conversion on LAI compared with 3 of 45 on placebo in the double-blind phase of the trial, and 6 of the 11 patients maintained culture-negative samples through the open-label and follow-up phase of the study. The majority of the subjects that achieved culture conversion were non-CF subjects with MAC infection [110]. Recently, Insmed initiated a Phase 3 clinical trial focused on studying LAI in non-CF MAC-infected patients together with standard of care (see https://clinicaltrials.gov/ct2/show/NCT02344004 - accessed 23rd May 2016).

Inhaled antibiotics have also been contemplated for *M. tuberculosis* infection [111]. The advantages of lung delivery, local targeting, avoidance of oral bioavailability issues and achievement of high drug concentrations are similar to those for other pulmonary infections with the added advantage of potentially targeting antibiotics directly to alveolar macrophages infected with *M. tuberculosis*. Several antibiotics have been tested in preclinical models using inhalable-formulation concepts including INH [112, 113], rifapentine [114], clofazimine [115] and levofloxacin [116], novel antibiotics [117], and drug combinations [118].

The most advanced inhaled antibiotic for *M. tuberculosis* treatment is a dry-powder form of capreomycin, a second-line polypeptide antibiotic given by injection with serious side effects that limit its long-term use [119]. Dry-powder capreomycin is manufactured by spray drying in particles containing >70% capreomycin and L-leucine to create particles with aerosol properties expected to result in efficient pulmonary drug delivery in both the bronchi and alveolar regions [120, 121]. In guinea pig pharmacokinetic (PK) studies, lung administration of capreomycin dry powder resulted in lower systemic exposure, longer half-lives and 50- to 100-fold higher lung concentrations than were obtained with IV or intramuscular (IM) routes of delivery [120, 121]. Notably, the lung concentrations following inhalation were considerably higher than the MIC of capreomycin against *M. tuberculosis* [122]. In a Phase 1 safety study in healthy volunteers, doses of dry-powder capreomycin of up to 300 mg were administered using a capsule-based dry-powder system. Delivery was generally safe and well tolerated, with transient post-dose cough observed as the primary adverse event [123]. Dose-proportional increases in plasma PK parameters were observed and the highest dose tested resulted in C_{max} levels that exceed the capreomycin MIC for

M. tuberculosis. While the high number of capsules (12×25 mg) required to achieve the potentially efficacious dose may be a significant limitation to further development, the capreomycin programme highlights the potential of the inhaled-antibiotic approach for *M. tuberculosis*.

17.6 Conclusion

Treatment paradigms established with inhaled antibiotics for *P. aeruginosa* suggest that early treatment to prevent or delay the establishment of chronic mycobacterial infection may be the most successful strategy to improve health. However, mycobacterial infections often present clinically when chronic infection already exists, making early eradication a challenge. In these cases, additional therapeutic interventions are needed to augment the activity of existing antibiotic regimens or to replace currently used antibiotics. In the case of pulmonary infections, direct delivery to the site of infection via inhalation is a logical approach that warrants more consideration. In recent years, a number of novel approaches and mechanisms have been described that may expand the clinical choices that physicians have to include antibiotics aimed at novel targets, but also target new aspects of mycobacterial growth such as biofilm formation and persistence (Figure 17.1). Hopefully, more concepts and compounds in early development are advanced towards clinical studies to both validate new concepts and create novel therapies that are broadly applicable to mycobacterial infections, including both *M. tuberculosis* and NTM.

References

[1] Lewis, K. (2013) Platforms for antibiotic discovery. *Nature Reviews Drug Discovery*, **12**, 371–387.
[2] Zumla, A., Nahid, P. and Cole, S.T. (2013) Advances in the development of new tuberculosis drugs and treatment regimens. *Nature Reviews Drug Discovery*, **12**, 388–404.
[3] Almeida Da Silva, P.E. and Palomino, J.C. (2011) Molecular basis and mechanisms of drug resistance in Mycobacterium tuberculosis: classical and new drugs. *Journal of Antimicrobial Chemotherapy*, **66**, 1417–1430.
[4] Yew, W.W., Lange, C. and Leung, C.C. (2011) Treatment of tuberculosis: update 2010. *European Respiratory Journal*, **37**, 441–462.
[5] WHO (2012) WHO Global Tuberculosis Report 2012.
[6] Thomson, R.M. and Yew, W.W. (2009) When and how to treat pulmonary non-tuberculous mycobacterial diseases. *Respirology*, **14**, 12–26.
[7] Cohen, N.R., Lobritz, M.A. and Collins, J.J. (2013) Microbial persistence and the road to drug resistance. *Cell Host & Microbe*, **13**, 632–642.
[8] Wakamoto, Y., Dhar, N., Chait, R. *et al*. (2013) Dynamic persistence of antibiotic-stressed mycobacteria. *Science*, **339**, 91–95.
[9] Aldridge, B.B., Fernandez-Suarez, M., Heller, D. *et al*. (2012) Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. *Science*, **335**, 100–104.
[10] Baek, S.H., Li, A.H. and Sassetti, C.M. (2011) Metabolic regulation of mycobacterial growth and antibiotic sensitivity. *PLoS Biology*, **9**, e1001065.
[11] Betts, J.C., Lukey, P.T., Robb, L.C. *et al*. (2002) Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Molecular Microbiology*, **43**, 717–731.
[12] Adams, K.N., Takaki, K., Connolly, L.E. *et al*. (2011) Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell*, **145**, 39–53.

[13] Gusarov, I., Shatalin, K., Starodubtseva, M. and Nudler, E. (2009) Endogenous nitric oxide protects bacteria against a wide spectrum of antibiotics. *Science*, **325**, 1380–1384.
[14] Shatalin, K., Shatalina, E., Mironov, A. and Nudler, E. (2011) H2S: a universal defense against antibiotics in bacteria. *Science*, **334**, 986–990.
[15] O'Toole, G., Kaplan, H.B. and Kolter, R. (2000) Biofilm formation as microbial development. *Annual Review of Microbiology*, **54**, 49–79.
[16] Bernier, S.P., Lebeaux, D., DeFrancesco, A.S. *et al*. (2013) Starvation, together with the SOS response, mediates high biofilm-specific tolerance to the fluoroquinolone ofloxacin. *PLoS Genetics*, **9**, e1003144.
[17] Moker, N., Dean, C.R. and Tao, J. (2010) Pseudomonas aeruginosa increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. *Journal of Bacteriology*, **192**, 1946–1955.
[18] Nguyen, D., Joshi-Datar, A., Lepine, F. *et al*. (2011) Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science*, **334**, 982–986.
[19] Vega, N.M., Allison, K.R., Khalil, A.S. and Collins, J.J. (2012) Signaling-mediated bacterial persister formation. *Nature Chemical Biology*, **8**, 431–433.
[20] Worlitzsch, D., Tarran, R., Ulrich, M. *et al*. (2002) Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. *Journal of Clinical Investigation*, **109**, 317–325.
[21] Hassett, D.J., Cuppoletti, J., Trapnell, B. *et al*. (2002) Anaerobic metabolism and quorum sensing by Pseudomonas aeruginosa biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. *Advanced Drug Delivery Reviews*, **54**, 1425–1443.
[22] Cambier, C.J., Falkow, S. and Ramakrishnan, L. (2014) Host evasion and exploitation schemes of Mycobacterium tuberculosis. *Cell*, **159**, 1497–1509.
[23] van der Wel, N., Hava, D., Houben, D. *et al*. (2007) M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells. *Cell*, **129**, 1287–1298.
[24] Falkinham, J.O., 3rd (2009) Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. *Journal of Applied Microbiology*, **107**, 356–367.
[25] Mullis, S.N. and Falkinham, J.O., 3rd (2013) Adherence and biofilm formation of Mycobacterium avium, Mycobacterium intracellulare and Mycobacterium abscessus to household plumbing materials. *Journal of Applied Microbiology*, **115**, 908–914.
[26] Feazel, L.M., Baumgartner, L.K., Peterson, K.L. *et al*. (2009) Opportunistic pathogens enriched in showerhead biofilms. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 16393–16399.
[27] Falkinham, J.O., 3rd, Iseman, M.D., de Haas, P. and van Soolingen, D. (2008) Mycobacterium avium in a shower linked to pulmonary disease. *Journal of Water and Health*, **6**, 209–213.
[28] Canetti, G. and Georgopoulos, G. (1955) [Isoniazid- and streptomycin resistance of Mycobacterium tuberculosis in tuberculous lesions of the lung treated with resection after chemotherapy. I. Frequency of resistance in chemotherapy with prolonged action; study of 285 cases]. *Revue de la Tuberculose*, **19**, 927–946.
[29] Lenaerts, A.J., Hoff, D., Aly, S. *et al*. (2007) Location of persisting mycobacteria in a Guinea pig model of tuberculosis revealed by r207910. *Antimicrobial Agents and Chemotherapy*, **51**, 3338–3345.
[30] Qvist, T., Pressler, T., Hoiby, N. and Katzenstein, T.L. (2014) Shifting paradigms of nontuberculous mycobacteria in cystic fibrosis. *Respiratory Research*, **15**, 41–46.
[31] Kerns, P.W., Ackhart, D.F., Basaraba, R.J. *et al*. (2014) Mycobacterium tuberculosis pellicles express unique proteins recognized by the host humoral response. *Pathogens and Disease*, **70**, 347–358.
[32] Hunter, R.L., Armitige, L., Jagannath, C. and Actor, J.K. (2009) TB research at UT-Houston – a review of cord factor: new approaches to drugs, vaccines and the pathogenesis of tuberculosis. *Tuberculosis*, **89** (Suppl. 1), S18–S25.
[33] Ojha, A. and Hatfull, G. (2012) Biofilms of Mycobacterium tuberculosis: New Perspectives of an Old Pathogen. In: *Deciphering the Secret Life of the Bacilli* (ed. P.-J. Cardona), Intech, pp. 181–192.

[34] Pang, J.M., Layre, E., Sweet, L. *et al*. (2012) The polyketide Pks1 contributes to biofilm formation in Mycobacterium tuberculosis. *Journal of Bacteriology*, **194**, 715–721.
[35] Sambandan, D., Dao, D.N., Weinrick, B.C. *et al.* (2013) Keto-mycolic acid-dependent pellicle formation confers tolerance to drug-sensitive Mycobacterium tuberculosis. *mBio*, **4**(3), e00222-13. doi: 10.1128/mBio.00222-13 7 May 2013.
[36] Ojha, A.K., Baughn, A.D., Sambandan, D. *et al*. (2008) Growth of Mycobacterium tuberculosis biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Molecular Microbiology*, **69**, 164–174.
[37] Greendyke, R. and Byrd, T.F. (2008) Differential antibiotic susceptibility of Mycobacterium abscessus variants in biofilms and macrophages compared to that of planktonic bacteria. *Antimicrobial Agents and Chemotherapy*, **52**, 2019–2026.
[38] Zambrano, M.M. and Kolter, R. (2005) Mycobacterial biofilms: a greasy way to hold it together. *Cell*, **123**, 762–764.
[39] Recht, J., Martinez, A., Torello, S. and Kolter, R. (2000) Genetic analysis of sliding motility in Mycobacterium smegmatis. *Journal of Bacteriology*, **182**, 4348–4351.
[40] Recht, J. and Kolter, R. (2001) Glycopeptidolipid acetylation affects sliding motility and biofilm formation in Mycobacterium smegmatis. *Journal of Bacteriology*, **183**, 5718–5724.
[41] Nessar, R., Reyrat, J.M., Davidson, L.B. and Byrd, T.F. (2011) Deletion of the mmpL4b gene in the Mycobacterium abscessus glycopeptidolipid biosynthetic pathway results in loss of surface colonization capability, but enhanced ability to replicate in human macrophages and stimulate their innate immune response. *Microbiology*, **157**, 1187–1195.
[42] Freeman, R., Geier, H., Weigel, K.M. *et al*. (2006) Roles for cell wall glycopeptidolipid in surface adherence and planktonic dispersal of Mycobacterium avium. *Applied and Environmental Microbiology*, **72**, 7554–7558.
[43] Yamazaki, Y., Danelishvili, L., Wu, M. *et al*. (2006) Mycobacterium avium genes associated with the ability to form a biofilm. *Applied and Environmental Microbiology*, **72**, 819–825.
[44] Ojha, A., Anand, M., Bhatt, A. *et al*. (2005) GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria. *Cell*, **123**, 861–873.
[45] Ghosh, S., Indi, S.S. and Nagaraja, V. (2013) Regulation of lipid biosynthesis, sliding motility, and biofilm formation by a membrane-anchored nucleoid-associated protein of Mycobacterium tuberculosis. *Journal of Bacteriology*, **195**, 1769–1778.
[46] Ojha, A.K., Trivelli, X., Guerardel, Y. *et al*. (2010) Enzymatic hydrolysis of trehalose dimycolate releases free mycolic acids during mycobacterial growth in biofilms. *Journal of Biological Chemistry*, **285**, 17380–17389.
[47] Grzegorzewicz, A E., Pham, H., Gundi, V.A. *et al*. (2012) Inhibition of mycolic acid transport across the Mycobacterium tuberculosis plasma membrane. *Nature Chemical Biology*, **8**, 334–341.
[48] Pacheco, S.A., Hsu, F.F., Powers, K.M and Purdy, G.E. (2013) MmpL11 protein transports mycolic acid-containing lipids to the mycobacterial cell wall and contributes to biofilm formation in Mycobacterium smegmatis. *Journal of Biological Chemistry*, **288**, 24213–24222.
[49] Wang, F., Sambandan, D., Halder, R. *et al*. (2013) Identification of a small molecule with activity against drug-resistant and persistent tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, E2510–E2517.
[50] Schorey, J.S. and Sweet, L. (2008) The mycobacterial glycopeptidolipids: structure, function, and their role in pathogenesis. *Glycobiology*, **18**, 832–841.
[51] Billman-Jacobe, H., McConville, M.J., Haites, R.E. *et al*. (1999) Identification of a peptide synthetase involved in the biosynthesis of glycopeptidolipids of Mycobacterium smegmatis. *Molecular Microbiology*, **33**, 1244–1253.
[52] Dubnau, E., Chan, J., Raynaud, C. *et al*. (2000) Oxygenated mycolic acids are necessary for virulence of Mycobacterium tuberculosis in mice. *Molecular Microbiology*, **36**, 630–637.
[53] Rachman, H., Strong, M., Ulrichs, T. *et al*. (2006) Unique transcriptome signature of Mycobacterium tuberculosis in pulmonary tuberculosis. *Infection and Immunity*, **74**, 1233–1242.
[54] Belisle, J.T., Vissa, V.D., Sievert, T. *et al*. (1997) Role of the major antigen of Mycobacterium tuberculosis in cell wall biogenesis. *Science*, **276**, 1420–1422.

[55] Sochorova, Z., Petrackova, D., Sitarova, B. *et al*. (2014) Morphological and proteomic analysis of early stage air-liquid interface biofilm formation in Mycobacterium smegmatis. *Microbiology*, **160**, 1346–1356.
[56] Cole, S.T. (2012) Infectious diseases: Transporter targeted in tuberculosis. *Nature Chemical Biology*, **8**, 326–327.
[57] Jain, M., Chow, E. and Cox, J. (2008) The MmpL protein family. In: *The Mycobacterial Cell Envelope* (eds M. Daffé and J.M. Reyrat), ASM Press, pp. 201–210.
[58] Tahlan, K., Wilson, R., Kastrinsky, C.B. *et al*. (2012) SQ109 targets MmpL3, a membrane transporter of trehalose monomycolate involved in mycolic acid donation to the cell wall core of Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*, **56**, 1797–1809.
[59] La Rosa, V., Poce, G., Canseco, J.O. *et al*. (2012) MmpL3 is the cellular target of the antitubercular pyrrole derivative BM212. *Antimicrobial Agents and Chemotherapy*, **56**, 324–331.
[60] Chen, P., Gearhart, J., Protopopova, M. *et al*. (2006) Synergistic interactions of SQ109, a new ethylene diamine, with front-line antitubercular drugs in vitro. *Journal of Antimicrobial Chemotherapy*, **58**, 332–337.
[61] Kim, J.H., O'Brien, K.M., Sharma, R. *et al*. (2013) A genetic strategy to identify targets for the development of drugs that prevent bacterial persistence. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 19095–19100.
[62] Mak, P.A., Rao, S.P., Ping Tan, M. *et al.* (2012) A high-throughput screen to identify inhibitors of ATP homeostasis in non-replicating Mycobacterium tuberculosis. *ACS Chemical Biology*, **7**, 1190–1197.
[63] Mikusova, K., Huang, H., Yagi, T. *et al*. (2005) Decaprenylphosphoryl arabinofuranose, the donor of the D-arabinofuranosyl residues of mycobacterial arabinan, is formed via a two-step epimerization of decaprenylphosphoryl ribose. *Journal of Bacteriology*, **187**, 8020–8025.
[64] Makarov, V., Manina, G., Mikusova, K. *et al*. (2009) Benzothiazinones kill Mycobacterium tuberculosis by blocking arabinan synthesis. *Science*, **324**, 801–804.
[65] Ojha, A. and Hatfull, G.F. (2007) The role of iron in Mycobacterium smegmatis biofilm formation: the exochelin siderophore is essential in limiting iron conditions for biofilm formation but not for planktonic growth. *Molecular Microbiology*, **66**, 468–483.
[66] Singh, P.K., Parsek, M.R., Greenberg, E.P. and Welsh, M.J. (2002) A component of innate immunity prevents bacterial biofilm development. *Nature*, **417**, 552–555.
[67] Moreau-Marquis, S., Bomberger, J.M., Anderson, G.G. *et al.* (2008) The DeltaF508-CFTR mutation results in increased biofilm formation by Pseudomonas aeruginosa by increasing iron availability. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, **295**, L25–L37.
[68] Banin, E., Vasil, M.L., and Greenberg, E.P. (2005) Iron and Pseudomonas aeruginosa biofilm formation. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 11076–11081.
[69] Berlutti, F., Morea, C., Battistoni, A. *et al*. (2005) Iron availability influences aggregation, biofilm, adhesion and invasion of Pseudomonas aeruginosa and Burkholderia cenocepacia. *International Journal of Immunopathology and Pharmacology*, **18**, 661–670.
[70] Chitambar, C.R. and Narasimhan, J. (1991) Targeting iron-dependent DNA synthesis with gallium and transferrin-gallium. *Pathobiology: Journal of Immunopathology, Molecular and Cellular Biology*, **59**, 3–10.
[71] Emery, T., (1986) Exchange of iron by gallium in siderophores. *Biochemistry*, **25**, 4629–4633.
[72] Yu, Q., Griffin, E.F., Moreau-Marquis, S. *et al*. (2012) In vitro evaluation of tobramycin and aztreonam versus Pseudomonas aeruginosa biofilms on cystic fibrosis-derived human airway epithelial cells. *Journal of Antimicrobial Chemotherapy*, **67**, 2673–2681.
[73] Kaneko, Y., Thoendel, M., Olakanmi, O. *et al*. (2007) The transition metal gallium disrupts Pseudomonas aeruginosa iron metabolism and has antimicrobial and antibiofilm activity. *Journal of Clinical Investigation*, **117**, 877–888.
[74] Hoffman, L.R. and Ramsey, B.W. (2013) Cystic fibrosis therapeutics: the road ahead. *Chest*, **143**, 207–213.
[75] Moreau-Marquis, S., O'Toole, G.A. and Stanton, B.A. (2009) Tobramycin and FDA-approved iron chelators eliminate Pseudomonas aeruginosa biofilms on cystic fibrosis cells. *American Journal of Respiratory Cell and Molecular Biology*, **41**, 305–313.

[76] Raad, I., Chatzinikolaou, I., Chaiban, G. *et al*. (2003) In vitro and ex vivo activities of minocycline and EDTA against microorganisms embedded in biofilm on catheter surfaces. *Antimicrobial Agents and Chemotherapy*, **47**, 3580–3585.
[77] Banin, E., Brady, K.M. and Greenberg, E.P. (2006) Chelator-induced dispersal and killing of Pseudomonas aeruginosa cells in a biofilm. *Applied and Environmental Microbiology*, **72**, 2064–2069.
[78] Allison, K.R., Brynildsen, M.P. and Collins, J.J. (2011) Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature*, **473**, 216–220.
[79] Peng, B., Su, Y.B., Li, H. et al. (2015) Exogenous Alanine and/or Glucose plus Kanamycin Kills Antibiotic-Resistant Bacteria. *Cell Metabolism*, **21**, 249–261.
[80] Conlon, B.P., Nakayasu, E.S., Fleck, L.E. *et al.* (2013) Activated ClpP kills persisters and eradicates a chronic biofilm infection. *Nature*, **503**, 365–370.
[81] Lu, T.K. and Collins, J.J. (2009) Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 4629–4634.
[82] Lu, T.K. and Collins, J.J. (2007) Dispersing biofilms with engineered enzymatic bacteriophage. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 11197–11202.
[83] Hatfull, G.F. (2014) Molecular Genetics of Mycobacteriophages. *Microbiology Spectrum*, **2**, 1–36.
[84] Schuster, M., Sexton, D.J., Diggle, S.P. and Greenberg, E.P. (2013) Acyl-homoserine lactone quorum sensing: from evolution to application. *Annual Review of Microbiology*, **67**, 43–63.
[85] Oppenheimer-Shaanan, Y., Steinberg, N. and Kolodkin-Gal, I. (2013) Small molecules are natural triggers for the disassembly of biofilms. *Trends in Microbiology*, **21**, 594–601.
[86] Davies, D.G. and Marques, C.N. (2009) A fatty acid messenger is responsible for inducing dispersion in microbial biofilms. *Journal of Bacteriology*, **191**, 1393–1403.
[87] Kolodkin-Gal, I., Romero, D., Cao, S. *et al*. (2010) D-amino acids trigger biofilm disassembly. *Science*, **328**, 627–629.
[88] Lam, H., Oh, D.C., Cava, F. *et al*. (2009) D-amino acids govern stationary phase cell wall remodeling in bacteria. *Science*, **325**, 1552–1555.
[89] Sanchez, C.J., Jr., Prieto, E.M., Krueger, C.A. *et al*. (2013) Effects of local delivery of D-amino acids from biofilm-dispersive scaffolds on infection in contaminated rat segmental defects. *Biomaterials*, **34**, 7533–7543.
[90] Moraes, G.L., Gomes, G.C., Monteiro de Sousa, P.R. *et al*. (2015) Structural and functional features of enzymes of Mycobacterium tuberculosis peptidoglycan biosynthesis as targets for drug development. *Tuberculosis*, **95**, 95–111.
[91] Wang, L.H., He, Y., Gao, Y. *et al*. (2004) A bacterial cell-cell communication signal with cross-kingdom structural analogues. *Molecular Microbiology*, **51**, 903–912.
[92] Deng, Y., Lim, A., Lee, J. *et al*. (2014) Diffusible signal factor (DSF) quorum sensing signal and structurally related molecules enhance the antimicrobial efficacy of antibiotics against some bacterial pathogens. *BMC Microbiology*, **14**, 51–59.
[93] Overhage, J., Campisano, A., Bains, M. *et al*. (2008) Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infection and Immunity*, **76**, 4176–4182.
[94] Mansour, S.C., de la Fuente-Nunez, C. and Hancock, R.E. (2015) Peptide IDR-1018: modulating the immune system and targeting bacterial biofilms to treat antibiotic-resistant bacterial infections. *Journal of Peptide Science*, **21**, 323–329.
[95] Rivas-Santiago, B., Castaneda-Delgado, J.E., Rivas Santiago, C.E. *et al*. (2013) Ability of innate defence regulator peptides IDR-1002, IDR-HH2 and IDR-1018 to protect against Mycobacterium tuberculosis infections in animal models. *PLoS One*, **8**, e59119.
[96] de la Fuente-Nunez, C., Reffuveille, F., Haney, E.F. *et al*. (2014) Broad-spectrum anti-biofilm peptide that targets a cellular stress response. *PLoS Pathogens*, **10**, e1004152.
[97] Klinkenberg, L.G., Lee, J.H., Bishai, W.R. and Karakousis, P.C. (2010) The stringent response is required for full virulence of Mycobacterium tuberculosis in guinea pigs. *Journal of Infectious Diseases*, **202**, 1397–1404.
[98] Paone, G., Stevens, L.A., Levine, R.L. *et al*. (2006) ADP-ribosyltransferase-specific modification of human neutrophil peptide-1. *Journal of Biological Chemistry*, **281**, 17054–17060.

[99] Paone, G., Wada, A., Stevens, L.A. *et al*. (2002) ADP ribosylation of human neutrophil peptide-1 regulates its biological properties. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 8231–8235.

[100] Stevens, L.A., Barbieri, J.T., Piszczek, G. *et al*. (2014) Nonenzymatic conversion of ADP-ribosylated arginines to ornithine alters the biological activities of human neutrophil peptide-1. *Journal of Immunology*, **193**, 6144–6151.

[101] Shteinberg, M. and Elborn, J.S. (2015) Use of inhaled tobramycin in cystic fibrosis. *Advances in Therapy*, **32**, 1–9.

[102] Ramsey, B.W., Pepe, M.S., Quan, J.M. *et al*. (1999) Intermittent administration of inhaled tobramycin in patients with cystic fibrosis. Cystic Fibrosis Inhaled Tobramycin Study Group. *New England Journal of Medicine*, **340**, 23–30.

[103] Geller, D.E., Weers, J. and Heuerding, S. (2011) Development of an inhaled dry-powder formulation of tobramycin using PulmoSphere technology. *Journal of Aerosol Medicine and Pulmonary Drug Delivery*, **24**, 175–182.

[104] Waters, V. and Smyth, A. (2015) Cystic fibrosis microbiology: Advances in antimicrobial therapy. *Journal of Cystic Fibrosis*, **14**, 551–560.

[105] Esther, C.R., Jr., Esserman, D.A., Gilligan, P. *et al*. (2010) Chronic Mycobacterium abscessus infection and lung function decline in cystic fibrosis. *Journal of Cystic Fibrosis*, **9**, 117–123.

[106] Floto, R.A. and Haworth, C.S. (2015) The growing threat of nontuberculous mycobacteria in CF. *Journal of Cystic Fibrosis*, **14**, 1–2.

[107] Griffith, D.E. (2007) Therapy of nontuberculous mycobacterial disease. *Current Opinion in Infectious Diseases*, **20**, 198–203.

[108] Waters, V. and Ratjen, F. (2014) Inhaled liposomal amikacin. *Expert Review of Respiratory Medicine*, **8**, 401–409.

[109] Meers, P., Neville, M., Malinin, V. *et al*. (2008) Biofilm penetration, triggered release and in vivo activity of inhaled liposomal amikacin in chronic Pseudomonas aeruginosa lung infections. *Journal of Antimicrobial Chemotherapy*, **61**, 859–868.

[110] Olivier, K., Gupta, R., Eagle, G. *et al*. (2014) Randomized, Double-Blind, Placebo-Controlled Study and Open-Label Extension of Liposomal Amikacin for Inhalation (LAI) in Patients with Recalcitrant Nontuberculous Mycobacterial Lung Disease (NTM-LD). Poster # 293 presented at the North American Cystic Fibrosis Conference, October 9–11, 2014, Atlanta, GA, USA.

[111] Hickey, A.J., Misra, A. and Fourie, P.B. (2013) Dry powder antibiotic aerosol product development: inhaled therapy for tuberculosis. *Journal of Pharmaceutical Sciences*, **102**, 3900–3907.

[112] Parikh, R., Dalwadi, S., Aboti, P. and Patel, L. (2014) Inhaled microparticles of antitubercular antibiotic for in vitro and in vivo alveolar macrophage targeting and activation of phagocytosis. *Journal of Antibiotics*, **67**, 387–394.

[113] Rojanarat, W., Changsan, N., Tawithong, E. *et al*. (2011) Isoniazid proliposome powders for inhalation – preparation, characterization and cell culture studies. *International Journal of Molecular Sciences*, **12**, 4414–4434.

[114] Chan, J.G., Duke, C.C., Ong, H.X. *et al*. (2014) A novel inhalable form of rifapentine. *Journal of Pharmaceutical Sciences*, **103**, 1411–1421.

[115] Verma, R.K., Germishuizen, W.A., Motheo, M.P. *et al*. (2013) Inhaled microparticles containing clofazimine are efficacious in treatment of experimental tuberculosis in mice. *Antimicrobial Agents and Chemotherapy*, **57**, 1050–1052.

[116] Rojanarat, W., Nakpheng, T., Thawithong, E. *et al*. (2012) Levofloxacin-proliposomes: opportunities for use in lung tuberculosis. *Pharmaceutics*, **4**, 385–412.

[117] Sung, J.C., Garcia-Contreras, L., Verberkmoes, J.L. *et al*. (2009) Dry powder nitroimidazopyran antibiotic PA-824 aerosol for inhalation. *Antimicrobial Agents and Chemotherapy*, **53**, 1338–1343.

[118] Chan, J.G., Chan, H.K., Prestidge, C.A. *et al*. (2013) A novel dry powder inhalable formulation incorporating three first-line anti-tubercular antibiotics. *European Journal of Pharmaceutics and Biopharmaceutics*, **83**, 285–292.

[119] Caminero, J.A., Sotgiu, G., Zumla, A. and Migliori, G.B. (2010) Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. *The Lancet Infectious Diseases*, **10**, 621–629.

[120] Garcia-Contreras, L., Fiegel, J., Telko, M.J. *et al*. (2007) Inhaled large porous particles of capreomycin for treatment of tuberculosis in a guinea pig model. *Antimicrobial Agents and Chemotherapy*, **51**, 2830–2836.

[121] Fiegel, J., Garcia-Contreras, L., Thomas, M. *et al*. (2008) Preparation and in vivo evaluation of a dry powder for inhalation of capreomycin. *Pharmaceutical Research*, **25**, 805–811.

[122] Garcia-Contreras, L., Muttil, P., Fallon, J.K. *et al*. (2012) Pharmacokinetics of sequential doses of capreomycin powder for inhalation in guinea pigs. *Antimicrobial Agents and Chemotherapy*, **56**, 2612–2618.

[123] Dharmadhikari, A.S., Kabadi, M., Gerety, B. *et al*. (2013) Phase I, single-dose, dose-escalating study of inhaled dry powder capreomycin: a new approach to therapy of drug-resistant tuberculosis. *Antimicrobial Agents and Chemotherapy*, **57**, 2613–2619.

18

Directed Intervention and Immunomodulation against Pulmonary Tuberculosis

Dominique N. Price and Pavan Muttil

Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico, Albuquerque, NM, USA

18.1 Introduction

Tuberculosis (TB) statistics are recited often among the research community because they are bleak and have remained largely unchanged for the last few decades. Over one third of the world population is infected with *Mycobacterium tuberculosis* (Mtb), and disease-associated mortality rates are the second highest of any infectious disease worldwide. At present, there is no effective vaccine, and drug discovery has been largely unproductive for the last 50 years.

However, TB is not the only disease associated with discouraging statistics. Many infectious and non-infectious diseases are difficult to treat and/or prevent. These illnesses tend to share one deadly characteristic, they hide from the host immune system. Cancer is perhaps one of the greatest challenges to the medical community. The body does not recognize and reject tumors as foreign invaders because tumors are host cells, replicating without the proper checkpoints. Researchers struggle to develop ways to target cancer cells and tumors, without harming the rest of the body. Using a pathogen as an example, the human immunodeficiency virus (HIV) hides from the immune system of the host by infecting vital immune cells, making it nearly impossible to treat. Researchers have spent billions of dollars trying to treat these diseases with conventional and novel therapies.

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

More recently, research efforts in these fields have shifted from commonly used therapeutics (such as antibiotics, chemotherapy and radiation) to different immunotherapies and vaccines [1–5]. The human immune system is both powerful and exquisite in terms of specificity. In theory, it can be primed, modulated, and/or tricked into responding to almost any material, with the caveat that many of the mechanisms by which this modulation of the immune system can be achieved are still poorly understood. In this chapter we explore efforts to modulate the immune system for the treatment of or immunization against TB. We will examine what has been successful and unsuccessful in the past, current research efforts, and speculate on were the field needs to go.

18.2 TB Immunology

18.2.1 Early Events of Infection

Mtb infection is predominantly acquired via the pulmonary route. An infected host with high bacterial burden and ruptured lung lesions releases droplets containing bacilli into the air (by coughing, sneezing, etc.) [6, 7]. Breathing in as few as one of these small droplets is enough to cause infection, with the infectious dose being less than 10 bacteria [6]. Once the bacilli have gained entry into the lung airway and parenchyma, the initial events of infection and the interplay between Mtb and the host are complex. Mtb has developed a multitude of mechanisms for exploiting the host immune response in order to further its own survival and spread.

The first stage of infection is mediated almost entirely by the host innate immune system. The mycobacteria are engulfed by phagocytic immune cells, upon activation of toll-like receptors (TLR) 2, TLR 9, and other pathogen-recognition receptors (including c-type lectin receptors and mannose receptors) present in these immune cells [7–10]. Phagocytic activity and initial inflammation lead to early granuloma formation. Classically, granuloma formation was thought to "wall off" the pathogen, in an effort to diminish bacterial replication while the adaptive arm of the immune system gained momentum. However, more recently, research has demonstrated a bacterial advantage in early infection of phagocytic cells [7, 11–15]. Alveolar macrophages, dendritic cells (DCs), and neutrophils are reservoirs by which Mtb can hide from adaptive immune cells [10, 11, 13]. This is illustrated by research showing that adaptive transfer of antigen-specific T cells during early infection does little to decrease bacterial numbers [10, 16]. Activated macrophages release interferon-gamma (IFN-γ) which initiates autophagy, wherein the macrophage phagosome (containing the bacterium) fuses with the lysosome, which increases compartment acidity and bacterial death [10, 17]. In addition, activated macrophages can control infection via apoptosis and efferocytosis (the phagocytosis of infected cells) [10, 12–14, 18].

However, Mtb manipulates macrophage recruitment into the lung, as well as delaying antigen presentation in the draining lymph nodes. Mtb has a complex lipid-rich cell wall that is important for cell survival as well as for host–pathogen interactions. These cell wall lipids limit antigen recognition and activation of macrophages, while promoting the recruitment of permissive macrophages to further infection [7, 10, 19]. In these macrophages Mtb replicates, preventing fusion of the phagosome and lysosome, as well as preventing apoptosis. Eventually the macrophage dies via necrosis, which is characterized by complete cell lysis, releasing bacteria and causing further spread of infection [10, 12, 14].

18.2.2 Delayed Adaptive Immunity

The increased necrosis of immune cells and dissemination of the bacteria limits cross-presentation of Mtb antigens by DCs in the draining lymph nodes, thereby delaying the adaptive immune response [10, 20]. The migration of DCs to the draining lymph node takes place 7–9 days after the initial infection. During this time the disseminating bacteria migrate out of the lung to form other lesions in the body. These extra-pulmonary lesions are efficiently cleared by splenic T cells most of the time. DCs take an additional 7–10 days to migrate from the draining lymph nodes back to the lung tissue, thus allowing Mtb up to 21 days to establish infection in the lung [7, 8, 10, 20]. Even when antigen-specific IFN-γ-producing T cells are present in the lung tissue (after parenteral vaccination or adoptive T cell transfer), control of bacterial replication and infection is only accelerated by 5–7 days [7, 8, 10]. Adaptive lymphocytes are essential to control of bacterial burden. T helper (CD4+) cells are especially critical. Mice deficient in functional CD4+ T helper cells cannot control bacterial replication and develop severe disease [8, 10, 20–22]. Similarly, HIV patients with decreased CD4+ T helper cells are at high risk for active TB and have increased TB morbidity and mortality. Despite the necessity of CD4+ T helper cells, the efficacy of these cells is temporally and spatially dependent, making it variable from patient to patient.

Many events may contribute to the delay of the adaptive immune response, which is critical for clearing Mtb. In addition to the aforementioned immune cell necrosis and subsequent bacterial dissemination, $FoxP3^+$ CD4+ T regulatory (Treg) cells and the cytokine interleukin 10 (IL-10) may directly inhibit and delay T helper cell recruitment to the site of infection [8, 10]. This delay may also be a mechanism of reduced phagocyte–lymphocyte contact. The long migration time of T cells from the circulation back to the lung parenchyma allows innate immune cells to continue to accumulate at the site of infection. Neutrophils and permissive macrophages interfere with the critical interaction between CD4+ T cells and infected macrophages, and decreases T cell effectiveness at controlling bacterial burden [7, 10].

CD8+ T cells are also involved in the adaptive response to TB, although not to the same extent as are CD4+ T cells. In fact the extent of their contribution to bacterial control is somewhat controversial in the literature. CD8+ T cells migrate along with CD4+ T cells into the lung parenchyma. Here they mediate control of infection via two pathways; (1) secretion of Th1 cytokines to recruit and activate phagocytes, and (2) the secretion of granulysin for the direct killing of infected phagocytes [10, 23]. However, CD8+ T cells alone are not sufficient to control infection. Sud and colleagues demonstrated this by depleting mice of CD4+ T cells, and inducing CD8+ T cells to produce enough IFN-γ to match wild-type levels of IFN-γ; however, CD8+ T cells were not sufficient to control infection by themselves [23]. In addition, when mice were either depleted of CD4+ T cells or IFN-γ, they had earlier active disease progression [23]. This is also seen in acquired immune deficiency syndrome (AIDS) patients with TB co-infection, as their CD8+ T cells are not sufficient to clear the disease.

18.2.3 Humoral Immunity and Innate Lymphocytes

The benefits of humoral immunity in TB are widely debated. Considered ineffective in controlling infectious burden early on in TB research, studies in this area were neglected for years [10, 20, 24–27]. However, the limited protection provided by bacille Calmette–Guérin (BCG) worldwide and the failure of vaccine competitors in clinical trials has re-invigorated

humoral research as a possible supportive mediator of TB immunity. Studies in B cell-deficient mice have shown varied results ranging from increased susceptibility and diminished protection, to reduced pathogenesis and dissemination, to no effect [27–32]. It is known that follicle-like B cell aggregates, or inducible bronchus-associated lymphoid tissues, are found in outer portions of the pulmonary granuloma in chronic TB infection [20, 27, 33]. While their function in TB still remains speculative, studies in mice challenged with pulmonary influenza virus have shown these lymphoid-like structures are important mediators of protective immunity and lymphocyte memory responses in mice lacking secondary lymphoid tissue [27, 34, 35]. In addition, while B cell-deficient mice challenged with low-dose Mtb aerosol appear to be unaffected by the loss of humoral immunity, high doses of aerosol or intravenous (IV) Mtb infection in B cell-deficient mice produced increased lung pathology and disease susceptibility [20, 28, 32]. Furthermore, B cells have been shown to modulate infection via the Fc-gamma receptors (FcγR), where selective antibody engagement of the stimulatory FcγR or inhibitory FcγRIIB on antigen presenting cells (APCs) results in an increased Th1 response and bacterial containment or IL-10 production and diminished protection, respectively [20, 27, 32]. These data taken together seem to indicate that B cell involvement in TB infection is more complicated than initially thought with many factors, including infectious dose, disease stage, and location of Mtb, affecting the role of humoral immunity.

Innate T cells are the newest addition to the field of TB immunity. Mucosal associated invariant T (MAIT) cells, invariant natural killer T (iNKT) cells, and gamma delta (γδ) T cells have all been shown to play a protective role in TB, although to what extent remains to be explored. MAIT cells are activated by the infection of airway epithelial cells [7, 36]. Activated MAIT cells can limit early bacterial growth and prevent infection progression. iNKT cells become activated during primary infection and secrete granulocyte-macrophage colony-stimulating factor (GM-CSF), which functions to recruit cytokine-producing immune cells into the lung [7, 37]. Similarly, γδ T cells recognize phosphoantigens on Mtb triggering rapid T cell clonal expansion. These cells secrete cytokines and can directly kill Mtb-infected cells and inhibit mycobacterial growth [7, 38].

18.2.4 Latent Infection

After infection with Mtb, disease progresses in two possible ways: (1) The patient develops active TB. Mtb replicates in the lungs, unperturbed by the immune system. Clinical symptoms develop including fever, weight loss, and flu-like symptoms. Within the lung, severe tissue damage occurs in areas of infection. (2) Alternatively, the patient develops latent TB and has no clinical symptoms of infection. With one-third of the world infected with Mtb, most individuals develop clinical latency, making the patient asymptomatic and possibly unaware of the infection. During latent infection the mycobacterium remains quiescent in the lungs, trapped in hypoxic granulomas and unable to replicate. Only 5–10% of individuals with latent TB progress to active disease during their lifetime, termed re-activation, and these individuals are usually immunocompromised. Individuals with latent TB will commonly test positive in a skin test. The skin test is performed by injecting purified protein derivative (PPD), or Mtb culture filtrate, directly beneath the top layer of skin. The induration that forms is measured, and is generally larger in individuals who have been exposed to the mycobacteria. Interestingly, individuals who are latently infected but who test

negative in the skin test are more likely to progress to active disease [39]. This is further complicated in individuals who are vaccinated with BCG and are PPD positive due to the immunization.

The diagnosis of latent TB infection is dependent on a positive skin test and interferon gamma release assay in the absence of active disease. This may be the result of bacterial clearance by the host immune system or granulomas in the lung preventing bacterial growth and disease progression. The granuloma structure is complex and multicellular. Fibrotic tissue surrounds the outside of the granuloma and the centre is formed by infected phagocytes, neutrophils, multinucleated giant macrophages, epithelioid cells, and foamy macrophages. A layer of lymphocytes and B cells surrounds the exterior of the granuloma [10, 40]. At the centre of the granuloma a caseous necrotic core develops due to cell lysis, creating a hypoxic environment where the bacteria remain dormant and unable to replicate.

Granulomas were originally thought to be static structures, trapping bacteria within the immune cell-comprised walls and preventing its dissemination. However, work with *Mycobacterium marinum* in a zebrafish TB model looking at innate immune cells, as well as a study looking at lymphocytes in TB granulomas in the liver of mice, showed that the granuloma is a dynamic structure with lymphocytes and macrophages moving throughout [26, 41–43]. Thus, the granuloma establishes an equilibrium with the host, with immune cells keeping the mycobacterial numbers constant and contained, while a small mycobacterial subpopulation continues to replicate [8, 44]. This idea of bacterial and immunological equilibrium in the host seems to support the clinical manifestation of extreme heterogeneity among granulomas, both within the lung and between patients.

18.2.5 Correlates of Protection and Tolerance

Until recently, the immune correlates of protection during Mtb infection were all pro-inflammatory, or Th1 differentiated. As mentioned earlier, CD4+ and CD8+ T lymphocytes are critical mediators of defense, as well as phagocytes and other innate inflammatory responders (see §18.2.1 *Early Events of Infection* and §18.2.2 *Delayed Adaptive Immunity*). IFN-γ and tumor necrosis factor α (TNFα) are two well-known pro-inflammatory cytokines present in the infected lung environment, which are critical for control of the infection, but have also been shown to have detrimental effects. IFN-γ is mainly secreted by CD4+ cells, in response to interleukin 12 (IL-12) secretion by Mtb-infected DCs. It can also be secreted by CD8+ T cells, iNKT cells, γδ T cells, and alveolar macrophages [10, 45–47]. Mice without IFN-γ are extremely susceptible to Mtb infection and morbidity [48, 49]. They cannot control bacterial growth, have defective macrophage activation and function, and fail to produce reactive nitrogen and oxygen species [10, 47–49]. In addition, IFN-γ is crucial to inhibit the secretion of possibly detrimental cytokines like IL-17, which recruits neutrophils into the lung [7]. However, despite its essential role in reducing bacterial burden, IFN-γ is a controversial correlate of protection. While the cytokine is produced in healthy PPD-positive patients, it has been shown that patients with active TB may have suppressed levels [47, 50–52]. This may be due to the ability of Mtb to inhibit IFN-γ signaling. This arguably makes IFN-γ levels a poor predictor of disease outcome alone [47].

TNFα has been shown to modulate granuloma formation, maintenance, and function [53, 54]. In conjunction with IFN-γ it mediates macrophage function and production of reactive nitrogen and oxygen species [47]. Mice treated with anti-TNFα antibodies exhibit

increased bacterial burden, disorganized granulomas, poor macrophage function and activation, and increased Treg numbers [8, 55–58]. However, despite the documented antimicrobial effects, it has been shown that TNFα is also associated with host-mediated lung pathology [47, 59–61].

More recently a role for anti-inflammatory cytokines and mediators has been implicated during TB infection; however, to what extent these cytokines are beneficial versus harmful remains unknown. Tregs, IL-10, and transforming growth factor β (TGFβ) are generally considered anti-inflammatory in nature, namely Th2- or regulatory-differentiated. Unrestrained inflammation can cause severe pathology in the lung, as mentioned above, so these anti-inflammatory cells and cytokines can be protective. FoxP3+ Tregs interact with CD4+ T cells and can limit their effector function and recruitment [10]. IL-10 de-activates macrophages, limits IFN-γ secretion, and suppresses CD4+ T cell and APC function [47, 62, 63]. TGFβ can also deactivate macrophages and suppress CD4+ T cell function, in addition to altering production of reactive nitrogen and oxygen species [47, 63–65]. However counterintuitive it may seem, these anti-inflammatory mediators may be essential in preventing severe lung pathology, as well as T cell exhaustion. Programmed cell death protein 1 (PD-1) is a cell-surface receptor expressed on activated B and T cells [10]. When it binds to its ligands, programmed death ligand 1 and 2 (PD-L1 and PD-L2), negative signal cascades inhibit cell proliferation and cytokine production [10]. PD-L1 is induced during chronic inflammation, as shown by increased PD-L1 levels in patients with active TB, compared with healthy or latently infected patients [10, 66]. Therefore, it appears that anti-inflammatory immune mediators may have a role in preventing the detrimental effects of unchecked inflammation, although too much inhibition of inflammatory mediators may worsen disease.

18.2.6 Natural Immunity against TB Infection

Only 10% of Mtb-infected individuals develop active disease during their lifetime. Therefore, we can assume the remaining 90% of the population has some form of natural immunity protecting them from developing active disease. This is the basis for vaccination strategies which use attenuated mycobacterial species or antigens in order to closely simulate natural immunity after Mtb infection. Our understanding of how natural immunity protects individuals following infection with Mtb is incomplete, despite a multitude of research exploring how the immune system responds to a TB infection. In addition, little is known about how accurately vaccine-induced immunity recapitulates natural immunity. Researchers know that natural immunity is not sufficient to provide protection against TB infection. This is evident in countries such as South Africa, where TB is highly endemic, suggesting that natural immunity does not provide protection in the majority of the population. Moreover, in successfully treated TB patients, previous disease constitutes a significant risk factor for future reinfection [67].

18.3 Animal Models of Immunotherapies and Vaccines for TB

Each animal model currently being used in TB research provides specific aspects of human disease pathology, though no single preclinical model provides the complete spectrum of human disease characteristics, i.e., immune response and lung pathology. The most

commonly used TB animal models include mouse, guinea pig, and non-human primates; the final choice of which animal model to use is often dependent on the cost, space, and the biosafety facilities available. Human TB progresses to different stages based on disease pathology and time of diagnosis: active TB, latent TB, or reactivated TB. Animal models for TB need to capture all or some of these aspects of different human disease stages. The focus of this section will be on the animal models used to study the immune response to immunotherapy pre- and post-Mtb exposure. A thorough description of other animal models is beyond the scope of this section and readers are encouraged to refer to more complete reviews on TB animal models [68].

18.3.1 Mouse Model

Mice are the most commonly used animal model to study novel vaccines and immunotherapies due to their low cost and ease of availability. In addition, the immunological reagents and tools required to study the responses to immunotherapies and vaccines are numerous and readily available for this animal species. The mouse model can also be easily manipulated to produce genetically modified strains [69]. Although mice are more resistant to TB infection compared with other animal models, including humans, the strong immune imprint after infection has allowed researchers to study the immune response to various immunomodulatory agents and therapies. However, the mouse model poorly mimics human lung pathology; they lack a well-organized granuloma, the immune structures that are known to harbor Mtb. Furthermore, these unorganized granulomas lack necrosis, fibrosis, and hypoxia [70], which are the hallmarks observed in human TB granulomas. Research efforts currently focus on developing mouse models that provide human-like pathophysiology in order to more accurately evaluate novel vaccines and therapies against TB [71–73]. This includes the C3HeB/FeJ mice (Kramnik model) that allow necrotic granuloma and hypoxic lesion formation in the lungs [74].

18.3.2 Guinea Pig Model

Guinea pigs have similar lung pathology to human TB patients and form well-structured granulomas after a low-dose experimental Mtb infection. However, guinea pigs are highly susceptible to experimental infection, unlike healthy humans; a few virulent organisms administered by the aerosol route can produce lung lesions, bacteremia, and even fatal disease. The major drawback of this animal model is the lack of widely available immunological reagents.

18.3.3 Non-human Primates Model

Non-human primates (NHPs) most closely recapitulate the clinical characteristics of human TB, but have the downside of being expensive, resource-intensive, and ethically controversial [75]. Cynomolgus macaques can develop either active disease or latent infection after aerosol infection based on gross pathology, bacterial counts, and histopathology [76]. Furthermore, the histological lesions in this animal model show thick fibrosis, mineralization, and central caseation similar to those observed in humans [75]. In addition, the granulomas are hypoxic [70]. NHPs show heterogeneity in lesion types when infected with low-dose Mtb, a clinical manifestation observed in TB-infected humans. However, the

NHP model is usually reserved for vaccine and therapy candidates that have shown remarkable success in small-animal models and are in an advanced stage to enter into clinical development. This is probably due to the significant cost and resources associated with this animal model [77, 78].

18.4 The Current TB Vaccine – Bacille Calmette Guérin

18.4.1 BCG Vaccine History

BCG is the only vaccine available against TB. It was produced more than a century ago from a virulent strain of *Mycobacterium bovis* using continuous *in vitro* passage to attenuate the bacteria. Immediately after BCG's creation, the first human newborns were vaccinated in Europe in 1921, which led to significant protection against TB in young children [79]. BCG's success in Europe in the first half of the 20th century allowed it to expand to other TB-endemic areas, notably in Africa and South-East Asia. Since 1974 BCG has been part of the World Health Organization (WHO) Expanded Program on Immunization (EPI), and the estimated coverage in TB-endemic countries now exceeds 80% [80]. The growing worldwide demand for this live vaccine led to different BCG strains with minor genetic differences being cultured and passaged around the world. These strains were later classified as "early" strains (Russia, Moreau, Japan, etc.) based on few gene deletions, and "late" strains (Tice, Danish, Pasteur, etc.) that acquired further deletions due to the different culture mediums and growth conditions used during BCG's manufacturing process [81]. Currently, more than 13 different BCG sub-strains have been reported [82]; however, none of these strains are effective against the most prevalent form of the disease, adult pulmonary TB.

18.4.2 Alternative Routes of BCG Delivery

The BCG vaccine was initially administered to children orally with milk after it was introduced in the early 1920s; a route that Calmette and Guérin thought was crucial for BCG's success. The oral route was discontinued after the Lubeck disaster in Germany, where 67 infants died after BCG vaccination. It was later recognized that the vaccine batch used to immunize infants in Lubeck was contaminated with virulent strains of Mtb [83]. This incident gradually led to the oral route of BCG delivery being replaced by the intradermal (ID) route, as the oral route was considered to be unsafe. In addition, the parenteral route that replaced the oral route required only one BCG dose, compared with 3 doses for oral immunization.

Since then, many other routes of BCG delivery have been attempted, including intranasal, pulmonary, as well as revisiting the oral route [84–90]. The goal for most of these studies was to target the mucosal immune system directly to provide better protection against pulmonary TB. Mittrucker *et al*. observed comparable levels of protection in mice against aerosol challenge with Mtb after oral and systemic BCG immunization [87]. However, different levels of dissemination and persistence of BCG were observed in various organs [87]. Others administered BCG orally after encapsulating the live bacteria to protect them against the harsh gastric environment [91]; encapsulated BCG administered by the oral route achieved viable bacteria in the mesenteric lymph nodes that correlated with protection against Mtb, compared with unencapsulated orally administered BCG [92]. However,

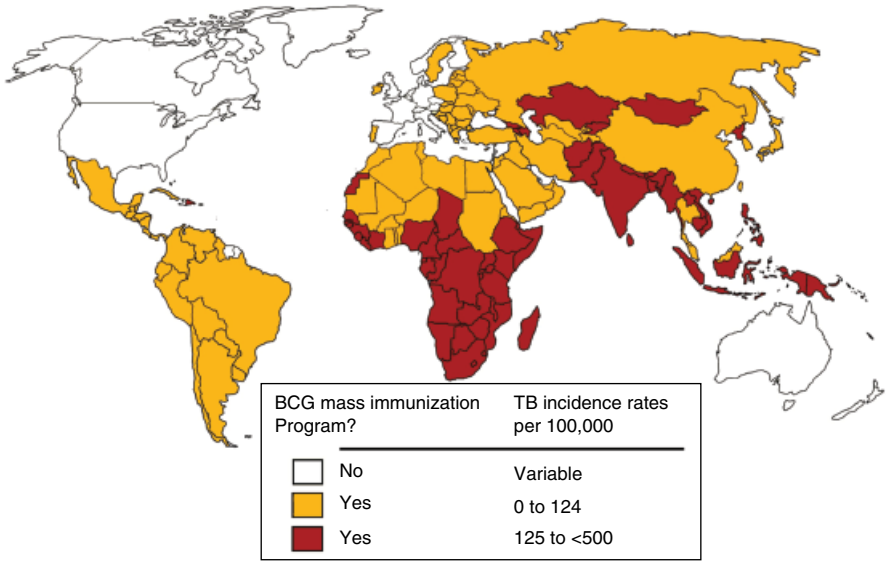


Figure 18.1 *BCG efficacy varies geographically around the world. Colored regions have National BCG Mass Immunization Programs in place, darker colors correspond to the highest TB burden regions. (See insert for color representation of the figure.)*

none of these delivery routes have succeeded in replacing the parenteral route of BCG administration that was adopted in the 1930s, in spite of the promising results obtained from alternative routes of BCG administration.

18.4.3 Failures of BCG

Despite immunization efforts in over 150 countries worldwide, BCG has failed to reduce disease burden in areas of the world where TB is endemic (Figure 18.1). A study in 1979 conducted by the WHO followed individuals in a controlled community trial in South India for $7^1/_2$ years and found no evidence of the protective effect of the BCG vaccine [93]. This was in contrast to initial trials in North America and the United Kingdom which showed 80% protection in vaccinated individuals [94, 95]. A later meta-analysis of the published literature showed that BCG provided an average of 50% protection, but the variability in efficacy rates could be largely explained by geographical location [96]. The various hypotheses for the inconsistent efficacy of BCG are as follows:

18.4.3.1 Non-tuberculosis Mycobacteria

Efforts to understand the inconsistency of BCG protection have been numerous, varied, and widely conflicting. One of the more common theories relates the varied geographical efficacy of the vaccine to the presence of high concentrations of environmental mycobacteria (EM) or non-tuberculosis mycobacteria (NTM) in areas where TB is endemic. NTMs, BCG, Mtb, and all other mycobacteria are remarkably alike, sharing similar protein-secretion pathways, cell-wall components, and even the similar 16S rRNA [97–99]. Initial NTM studies

were linked to the South India trials, where high levels of NTMs, specifically *Mycobacterium avium-intracellulare-scrofulaceum* (MAIS) complex, were isolated from the environment and sputum of vaccinated individuals with symptoms of TB infection [100]. Later these studies were validated in animal models of TB, showing that chronic NTM exposure results in the modulation of the host response and protective effects of BCG immunization [101–105].

Speculation on the underlying mechanistic reasons behind the modulation of host response to BCG after NTM exposure remains varied and uncertain. Circulating theories in the literature include the masking hypothesis and the blocking hypothesis, with evidence supporting both. The masking hypothesis was initially suggested in a study by Palmer and colleagues, which showed that pre-sensitization with NTMs offered some immunity against Mtb in guinea pigs, and later immunization with BCG conferred little to no additional anti-mycobacterial immune response [106, 107]. This was further illustrated by later research which compared pre-mycobacterial immunity, via delayed-type hypersensitivity (DTH) skin-test response to tuberculin PPD, of individuals in Malawi and the UK before and after BCG vaccination. The results showed that individuals in the UK started with much lower pre-existing immunity to mycobacteria, leading to a large increase in response after BCG vaccination. In contrast, individuals in Malawi had high pre-existing immunity to mycobacteria, leading to very little increase in immune response after BCG vaccination. The effect of prior NTM immunity "masked" the immunity gained by BCG vaccination, rendering BCG an ineffective booster of prior mycobacterial immunity (Figure 18.2B) [108, 109]. The blocking hypothesis was suggested in a study which looked at the replication of BCG in the lungs of vaccinated mice. Prior NTM exposure resulted in immunity significant enough to "block" BCG replication, but not sufficient to block Mtb growth, which was unchanged by NTM pre-sensitization [101]. This short-lived time-frame wherein BCG was present in the lungs was not considered sufficient enough to generate a strong immune response (Figure 18.2A).

Another hypothesis generated recently suggests that NTM exposure creates a tolerogenic immune response to mycobacteria in hosts. Oral tolerance is a widely accepted phenomenon mediated by the immune cells in the gut. Chronic oral exposure of antigens initiates immune cells in the gut mucosa to switch to a tolerogenic phenotype. By definition, oral tolerance is the lack of immune response to parenteral immunization after chronic oral exposure to the same antigen [110]. Muttil and colleagues have shown that classic NTM immune modulation appears to be exclusive to oral exposure. When mice were chronically exposed to NTMs by the oral and ID route, only orally pre-sensitized mice displayed a diminished immune response to BCG [111].

18.4.3.2 BCG Vaccine Antigenicity

NTM exposure is not the only explanation for poor BCG protection. Many researchers believe that the live vaccine itself is inconsistent. Since its initial origin in the laboratory of Calmette and Guérin in the early 1900s, *M. bovis* was passaged over 230 times before becoming the attenuated BCG first administered to humans in 1921. The vaccine continued to be passaged for maintenance because there was no cold chain storage or lyophilization. By the time the first BCG stock was freeze-dried, the Pasteur strain in 1961, it had been passaged over 1173 times [112, 113]. As mentioned before, over 13 well-studied strains of

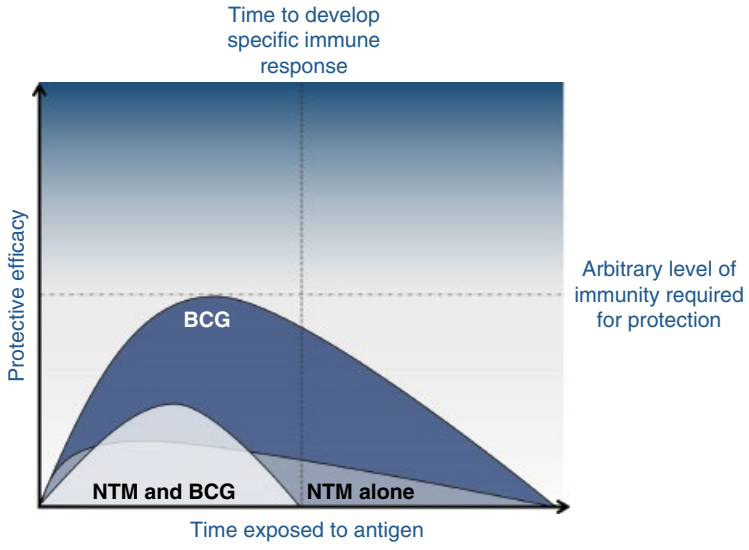


Figure 18.2A Blocking hypothesis. *BCG in naive individuals is able to replicate in the lungs long enough for the development of a BCG-specific immune response to develop. This level of immunity is protective against Mtb challenge (BCG curve). NTMs in naive individuals replicate and generate a NTM-specific immune response; however, this level of immunity is not sufficient for protection against subsequent Mtb infection (NTM alone curve). BCG in NTM-exposed individuals provides insufficient protective immunity against TB. Prior NTM immunity inhibits BCG replication and the generation of a BCG-specific immune response (NTM and BCG curve). (See insert for color representation of the figure.)*

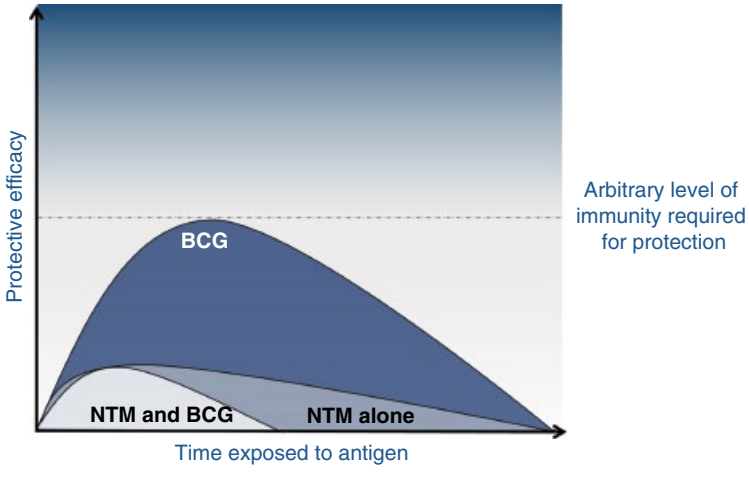


Figure 18.2B Masking hypothesis. *BCG in naive animals provides sufficient protection against Mtb challenge (BCG curve). NTM exposure in naive individuals generates some immunity, but this is insufficient against Mtb challenge (NTM alone curve). BCG in NTM-exposed individuals does not generate immunity above the immunity already generated by NTM exposure, leaving them susceptible to Mtb infection (NTM and BCG curve). (See insert for color representation of the figure.)*

BCG exist, and have different molecular and genetic fingerprints. Different geographical regions of the world use different strains of BCG, and while each daughter strain was originally produced to maintain attenuation as well as antigenicity, little is known about which strains are the most effective.

18.4.3.3 Mtb Strain Virulence

There are many different strains of Mtb that have branched off this ancient bacteria's phylogenetic tree and 6 main strains that infect different areas of the world [114, 115]. Gagneux and colleagues demonstrated that the host immune system can respond very differently to the different strains of Mtb. For example, it has been shown that ancient lineages of Mtb, when compared with more modern lineages, stimulate the innate immune response better. This may have evolved as a mechanism of virulence and spreading capability of the mycobacterium. Ancient lineages are associated with low-density areas of the world, and may have evolved such that a robust innate immune response may have been beneficial to the bacteria, allowing them to remain latent in their host for later spread. This is in contrast to more modern strains of TB, which infect population-dense areas of the world and seem to avoid robust innate host immunity. It has been speculated that these strains of Mtb suppress innate immune functions because spread in these population-dense areas is more likely. These strain-to-strain differences in eliciting the host immune response may be important to consider when thinking about global differences in BCG efficacy.

18.4.3.4 Helminths

Another popular hypothesis which attempts to explain the poor protection associated with BCG immunization is immune interference by helminth co-infection. Helminths share the same geographical niches as Mtb, with the highest infection rates in Africa, Asia, and South America. Helminths are an extremely diverse group of gastrointestinal parasites which are acquired when food and water sanitation is poor [116]. These parasites alter the immune environment of their host from a dominant Th1 response (pro-inflammatory and cell-mediated) to a Th2 response (lymphocyte-suppressive, mostly humoral immune response). These responses can cross-regulate each other; Th2 cytokines suppress the secretion of Th1 cytokines and thereby prevent the Th1 immune cell migration required to protect against Mtb [117].

18.5 Other Vaccines Platforms

18.5.1 Live Bacterial Vaccines

Live bacterial vaccines induce immune responses either to the bacteria or to a specific antigen expressed by the bacteria. They have the advantage of mimicking a natural infection, and eliciting a potent immune response to one or more of the expressed antigens. In addition, live vaccines have intrinsic adjuvant properties which stimulate a strong mucosal immune response [118, 119]. Live bacterial constructs are therefore an attractive vaccination strategy to enhance immunity against a pathogen. The survival of BCG vaccine in the host after immunization is necessary for optimum induction of the immune system [112, 120].

Furthermore, live BCG strains, compared with dead bacilli, are critical for protection in mice against Mtb infection [121, 122]. However, live BCG is also known to cause local and disseminated complications, especially in immunocompromised individuals. BCG sub-strains used in human vaccination differ in the percentage of viable bacteria present at the time of vaccination, their capacity to survive in the host after systemic administration, and their ability to produce antigens in the host [96, 112]. Yet no clinical trial has been conducted to specifically address the question of BCG survival in vaccinated individuals and their probability of contracting TB. The goal of any live bacterial vaccine is to be non-pathogenic to the host, but still elicit a long-lasting immune response. This could be achieved by i) *Attenuating* the pathogenic bacteria – this involves the deletion of essential virulence factors [123]. ii) Developing stable *auxotrophs* – these are bacteria that are not able to replicate, or have limited capacity for replication in the vaccinated individuals. iii) *Recombinant* bacterial strains that overexpress Mtb antigens or proteins that are known to generate a potent anti-mycobacterial immune response. These antigens work by different mechanisms; one of them is to allow the live vaccine to escape the phagolysosome into the cell cytoplasm to generate a strong CD8+ T cell response [124].

18.5.1.1 Safety Concerns of Live Vaccines

In spite of the above safety checkpoints of live bacterial vaccines, the risk of conversion from the avirulent to virulent state, thereby increasing the probability of causing disease, still exists. Other safety fears with live bacterial vaccines include systemic infection, dissemination to organs, induction of tolerance to a specific or related bacterial strain used for vaccination, potentiation of autoimmunity, gene/plasmid transfer to host's indigenous flora, suppression of the indigenous flora due to competition, and transfer of the live bacterial vaccine to other individuals.

Integration of a mycobacterial antigen-encoding plasmid into a live recombinant vaccine could be a potential hazard. It is possible that the altered vaccine strain could horizontally transfer the plasmid to other bacteria present in the vaccinated individual. This could lead to the undesirable persistence of the plasmid in the host. However, humans are constantly exposed to pathogenic and non-pathogenic bacteria without significant damage. These bacterial exposures usually happen by the oral and/or the pulmonary route, rather than the parenteral route by which most immunizations are administered. The route of vaccine administration could therefore be a critical factor when evaluating this specific hazard [125]. Many of the above safety concerns could be mitigated by the dose, regimen (single or multiple immunizations), and route of delivery. Although live vaccines continue to show significant promise in preclinical and early clinical trials, sufficient safety studies are required to gain the confidence of the world regulatory agency to move forward with any new live TB vaccine [126].

18.5.2 Inactivated Whole-cell Vaccines

The role of inactivated vaccines is to mimic a natural infection, allowing the human body to generate its own protective immunity, similar to that of a live-attenuated vaccine (discussed in § 18.5.1.1 *Safety Concerns of Live Vaccines*). However, unlike with live bacterial vaccines, there is no fear of dissemination or infection from an inactivated whole-cell vaccine. In a study by Vuola *et al*., five doses of inactivated *Mycobacterium vaccae* induced

durable cellular immune responses in HIV-positive individuals [127]; the local and systemic reactions to inactivated *M. vaccae* were milder and less frequent than those normally observed with live BCG, implicating it as a safer vaccination strategy [128]. Moreover, the T-cell responses in these HIV-infected patients were maximal in recipients who were earlier primed with childhood BCG vaccine [129], thus making inactivated whole-cell mycobacterial vaccine a well-tolerated prime-boost immunization strategy in HIV-infected persons [130]. However, the delay in implementing inactivated mycobacterial vaccines from clinical trials to real life could be due to the multiple-dose immunizations required to elicit a protective immune response, especially in immunocompromised individuals.

18.5.3 Viral Vector-based TB Vaccines

Viruses such as pox virus, adenovirus, flaviviruses, and lentiviruses have been used as vectors for antigen delivery in vaccines for various infectious diseases. Viral vector-based vaccines are known to generate robust cellular immunity. Recombinant adenoviral vectors are potent inducers of cellular immunity, especially human leukocyte antigen (HLA) class I-restricted CD8+ T cell responses [131]. For viral vector-based vaccines against TB, dominant Mtb subunits are expressed in these vectors and have been evaluated as heterologous boosters in BCG-primed individuals. Such vaccines have been used for both pre-exposure administration in infants and post-exposure administration in adults.

MVA85A/Aeras-485 vaccine is a modified vaccinia virus Ankara (MVA) construct expressing the immunodominant antigen Ag85A that is shared between both Mtb and BCG. MVA85A recently completed a phase 2b clinical trial in South Africa. This vaccine was given to 4–6-month-old infants as a boost to BCG prime given at birth. However, MVA85A failed to provide protection at a 2-year follow-up compared with the group that only received BCG vaccine [132]. Surprisingly, this vaccine induced all the known immune predictors of vaccine protection studied in preclinical animal models (including mice, guinea pigs, NHPs, and cattle) against TB [133–136]. These immune correlates included Mtb-specific CD4+ T cells and mononuclear cells producing IFN-γ; pro-inflammatory cytokines such as interleukin 2 (IL-2) and TNFα, and increased Mtb-specific TH-17 T cells. Furthermore, in these preclinical studies, the vaccine provided protection against TB. Unfortunately, the immunological correlates of protection generated in these small- and large-animal models could not be replicated in BCG-boosted infants outside a laboratory setting. It is possible that the immature and weak immune system of infants, which has yet to develop polyfunctional T cells against Mtb antigens, could not generate the robust response to immunization required to effectively protect against TB infection. Since the majority of individuals contract TB during their adolescent years when the immunity of BCG is waning, viral-vector-based vaccines should be further evaluated in an adult population.

In a phase 1 clinical trial in BCG-vaccinated South African adults, another viral-vectored vaccine, Crucell Ad35/Aeras-402, induced robust and polyfunctional CD4+ and CD8+ T cells [137]. Recently, Aeras-402 was also evaluated as an inhaled vaccine in NHPs prior to Mtb aerosol challenge [77]. Although the vaccine generated a robust cellular immune response in the lung compartment, it failed to provide protection against a high Mtb challenge dose. This study again showed the inconsistency between the immune correlates and the protection provided in the host. Adenoviral-based vaccines also exacerbated infection in HIV patients thus making them more susceptible to AIDS.

Therefore, the use of adenovirus-based vaccines in an HIV–TB co-infected patient needs to be reevaluated [138–140].

18.5.4 Heterologous Prime-boost Vaccination Strategy in TB

The boosting of immunity against infectious diseases during the lifetime of an individual is not a novel concept. Adults are more susceptible to TB, as BCG-induced protection wanes with time after childhood immunization. However, TB booster immunizations are ineffective in extending the protection afforded by BCG given at birth. TB vaccine development has therefore focused on developing i) a successful vaccine to boost individuals who were immunized with BCG in early childhood. This could improve the immunity in more than 2 billion adults with apparent latent TB infection to prevent future reactivation [141]. The risk of developing latent TB infection increases by more than 60% in immunized adults in TB-afflicted Sub-Saharan African countries [142]. ii) A strong prime immunization strategy that is receptive to multiple boosters during an individual's lifetime. This should include a prime childhood immunization with a vaccine expressing latent TB antigens and a booster vaccine, again consisting of latent antigens.

Subunit vaccines have been proposed as a booster to either BCG, recombinant BCG, or attenuated Mtb vaccine in order to maintain high levels of immunity in the roughly 10% of individuals vulnerable against contracting TB during their lifetime [143]. Subunit vaccines can be delivered either via viral vectors or as recombinant proteins mixed with adjuvants. Subunit vaccines in clinical trials include the M72 vaccine in AS01E adjuvant [144], and hybrid 56 (ESAT-6) and Ag85B fused with the dormancy antigen Rv2660 [145].

A number of heterologous prime-boost immunization approaches have been evaluated in animal models and human trials, with BCG given typically as the prime vaccine. The initial development of BCG from *M. bovis* led to the deletion of the Region of Difference (RD1) that encodes at least 9 antigens that are present in Mtb. Two of the early secretory proteins missing from BCG, ESAT-6 and CFP10, are recognized by almost all TB patients [146]. In addition, the non-replicating persistence of Mtb during a latent infection allows the mycobacteria to express and upregulate latency genes [147, 148]. BCG does not express these latency antigens since it does not enter into a state of dormancy after immunization, and therefore does not induce an immune response in the host to the latency antigens [149]. Attempts have been made to express some of these latency antigens in recombinant BCG [150]. These studies demonstrated long-term protection in an aerosol-challenged mouse model. Additionally, it has been shown that BCG-primed animals (including NHPs) that were boosted with subunit vaccines containing ESAT-6 had a strong protective immune response that resulted in subsequent protection against aerosol Mtb challenge [151, 152]. In a recent study, post-exposure immunization of mice with ESAT-6-containing subunit vaccines prevented reactivation of TB, whereas BCG-only immunization did not provide protection [153].

The timing of booster dose(s) delivery must also be considered. In animal studies, booster immunization is often performed quickly due to costs associated with animal housing. However, a boost immunization should be delivered after the host develops a memory immune response and should be timed based on trials in BCG-primed children [154].

The route of boost vaccination is also critical for generating a strong immune response in the host. It is evident that parenteral boosting with viral-vector-based vaccines has been

less effective than respiratory mucosal boosting. All TB candidate booster vaccines currently in clinical trials are being evaluated by the parenteral route [155]. However, parenteral boosting in BCG-primed infants was ineffective in a recent phase 2b trial [132].

18.6 Pulmonary Immunization

18.6.1 Biomimicry: Harnessing Natural Immunity for Protection against TB

According to Janine Benyus, biomimicry involves using elements of nature to solve complex human problems [156]. Biomimicry, in the realms of an immunization strategy, would consist of emulating nature by developing and administering vaccines that resemble natural infection (Figure 18.3). We have been using biomimicry in the field of immunization ever since Jenner developed a vaccine against cowpox. Natural exposure to such pathogens usually occurs by the pulmonary, nasal, gastrointestinal or reproductive mucosa. Therefore, using attenuated bacterial strains for immunization, without integrating the natural exposure route, may not be sufficient to generate a robust and long-lived immunity against a pathogen. Furthermore, using biomimicry to inspire immunization platforms ensures that there is protection at the port of pathogen entry [157].

18.6.2 Pulmonary Immunization for Global Protection

While hopes for global vaccination against TB have been fraught with disappointment, researchers have tried to change the route of immunization and/or add boosters to increase protection. The pulmonary route of immunization may alleviate many of the current

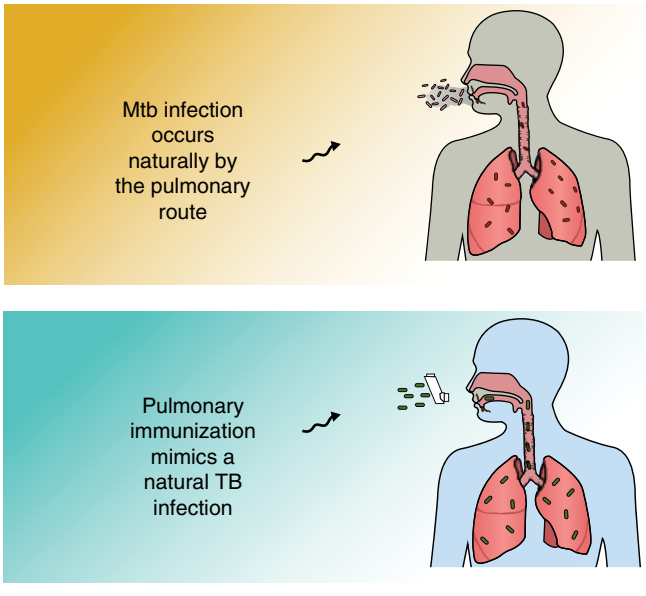


Figure 18.3 ***Biomimicry:*** *Pulmonary immunization with a live bacterial vaccine will mimic a natural infection and provide protection to the host. (See insert for color representation of the figure.)*

failures of BCG. It seems logical that the airway would provide the best tissue platform for vaccination as it is the entry site for Mtb infection, and vaccination via this delivery route would mimic infection elegantly [158]. Multiple studies have shown increased protective efficacy via the pulmonary route in various animal models of TB [77, 90, 111, 133, 159–162]. Garcia-Contreras *et al*. examined BCG vaccination efficacy by aerosol and showed a 2-log reduction of Mtb burden in the lungs of guinea pigs compared with ID vaccination alone [88]. Even more supportive is research published by Xing and colleagues comparing intranasal immunization with intermuscular immunization. Intramuscular vaccination failed to generate immune cells in the airway of the lungs of mice, generating only adaptive immunity in the systemic lymph tissues and lung parenchymal tissue. Only immunization via the airway was able to generate immune cells in the airways of mice, which was protective upon subsequent Mtb challenge (Figures 18.4A and 18.4B) [86, 157, 163, 164].

Muttil and colleagues recently showed that pulmonary vaccination may be the only means of vaccinating individuals living in NTM-endemic regions of the world [111]. These studies showed that mice exposed to NTMs via the gastrointestinal tract (mimicking chronic oral NTM exposure in humans) became tolerant or non-responsive immunologically to mycobacterial exposure, including BCG. This oral tolerance had a systemic effect, manifesting itself throughout the body. However, the immunologically-naive airway was unaffected by these immunosuppressive NTMs, making it the perfect route for vaccination. In a challenge model of mice exposed to chronic NTMs by the oral route, only pulmonary-vaccinated mice were able to generate immune cells in the airway and had more than a log reduction in Mtb CFUs in the lung compared with ID-vaccinated NTM-exposed mice [111].

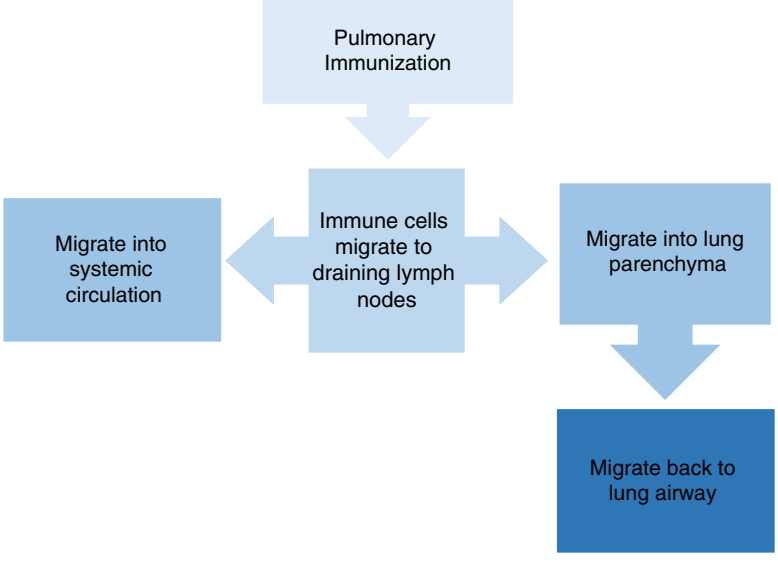


Figure 18.4A *Pulmonary immunization will generate protective immune cells in the airways and provide protection from aerosol Mtb challenge*

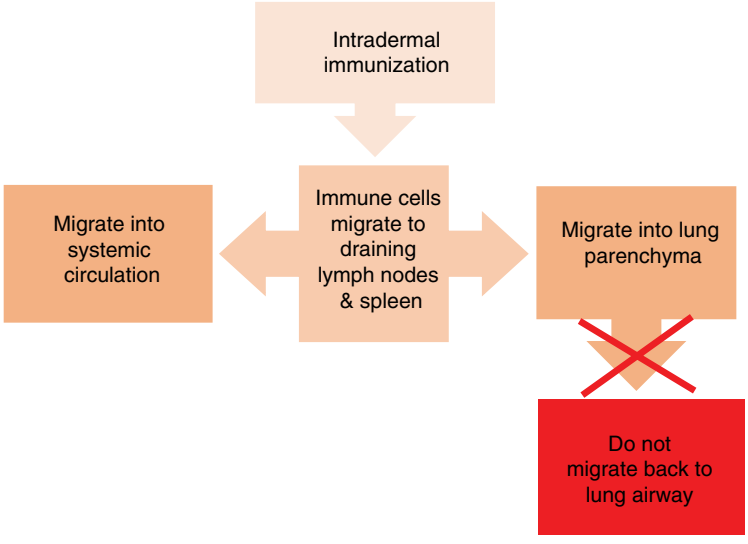


Figure 18.4B *Existing parenteral immunization with BCG or new vaccines utilizing the parenteral route will fail to generate protective immune cells in the airways*

In addition, pulmonary vaccination has the added benefit of eliciting localized immunity in the form of resident memory T cells (T_{RM}). Multiple groups have shown that DCs from specific tissues have the ability to impart tissue-specific homing chemokines to T cells, such that they will reside and home to the specific tissue where they first encountered the antigen [161, 165–167]. Antigen-specific T cells migrate to inflammation regardless of origin, but TB is associated with a notably long delay in T cell migration into the lungs (see § 18.2.2 *Delayed Adaptive Immunity*). Having T_{RM} cells present in the lung when infection begins may increase the speed in which T cells respond to the actively multiplying mycobacterium [161].

18.6.3 Safety Concerns for Pulmonary Immunization

As mentioned above, pulmonary immunization against TB has been evaluated for feasibility and efficacy studies in animal models and has shown better protection compared with parenteral immunization. However, pulmonary immunization against TB has not been able to cross regulatory hurdles due to the risk associated with this delivery route. Recently, a phase 1 safety and immunogenicity study was undertaken in BCG-vaccinated healthy adults with the MVA85A vaccine delivered as aerosol [78]. In this study, the pulmonary route was well-tolerated with only mild respiratory adverse events observed. Future studies should include a larger cohort of individuals to assess the pulmonary route of vaccination.

18.6.4 Role of Adjuvants

There is a need for the development of effective and safe adjuvants that can be delivered by the pulmonary route. Very few adjuvants are currently used clinically, and none of

them are approved for pulmonary delivery. The adjuvants used for parenteral vaccines, including alum, are not deemed to be safe for pulmonary delivery [168]. Muramyl dipeptide and trehalose dibehenate were evaluated in *in vitro* studies for use as an adjuvant in inhaled TB vaccines [169]. However, many pulmonary immunization studies use vaccines that have inherent adjuvant properties, including live bacterial and viral vector-based vaccines.

18.6.5 Live vs Dead Vaccines

The safety concerns of live vaccines administered by the parenteral route are discussed in an earlier section (see § 18.5.1.1 *Safety Concerns of Live Vaccines*). Delivering a live vaccine by the pulmonary route could lead to dissemination and disease, especially in immunocompromised individuals. However, different doses of live BCG were safely administered by the pulmonary route in cancer patients without any major adverse effects [170]. BCG was well-tolerated in metastatic lung cancer patients, with symptoms consisting of transient chills for 4–8 hours post-BCG aerosol delivery. In addition, rhesus macaques were vaccinated by the pulmonary route with a replication-defective recombinant adenovirus (rAd; AERAS-402) expressing the Mtb antigens, Ag85A and TB10.4 [77]. The rAd is a safe vaccine-delivery vector due to its non-replicating nature and was suited for pulmonary immunization due to its natural tropism for the respiratory tract [171].

18.7 Immunotherapeutic Agents against TB

The poor TB control and the growing TB–HIV co-epidemic continue to fuel the emergence of multidrug-resistant (MDR)- and extensively drug-resistant (XDR)-TB. The treatment for MDR strains involves prolonged treatment with first- and second-line anti-TB drugs. XDR strains are resistant to a fluoroquinolone and at least one second-line injectable drug, along with isoniazid and rifampicin (which constitute MDR resistance). MDR-TB has recently been detected in up to 35% of newly diagnosed patients in certain regions of the world. Furthermore, 76.5% of patients treated previously with anti-TB drugs revisit the hospital with a resistant strain of TB [67].

Only two new TB drugs have been introduced in the last 50 years, bedaquiline and delamanid, which are indicated for the treatment of MDR- and XDR-TB exclusively. We are currently fighting a losing battle between the development of new drugs and the co-evolution of Mtb strains that could soon become resistant to these antimicrobial agents (Figure 18.5). The development of alternative therapies against the growing pandemic of drug-resistant TB is urgently required. Immunotherapy alone, or as an adjunct to the existing anti-TB drugs, could be critical in controlling the spread of drug-resistant Mtb strains. In patients with MDR- and XDR-TB, for whom the standard anti-TB therapy is ineffective, immunotherapy either alone or in combination with existing anti-TB drugs, may be the only way to improve treatment outcomes. In addition, the combination of immunotherapy with chemotherapy could shorten the treatment regimen in these patients. Certain cytokines are already in clinical use as immune-modulators for TB and are discussed in the next section. The known safety profile of such immunotherapeutic agents in humans could allow their quick approval for widespread use in TB patients.

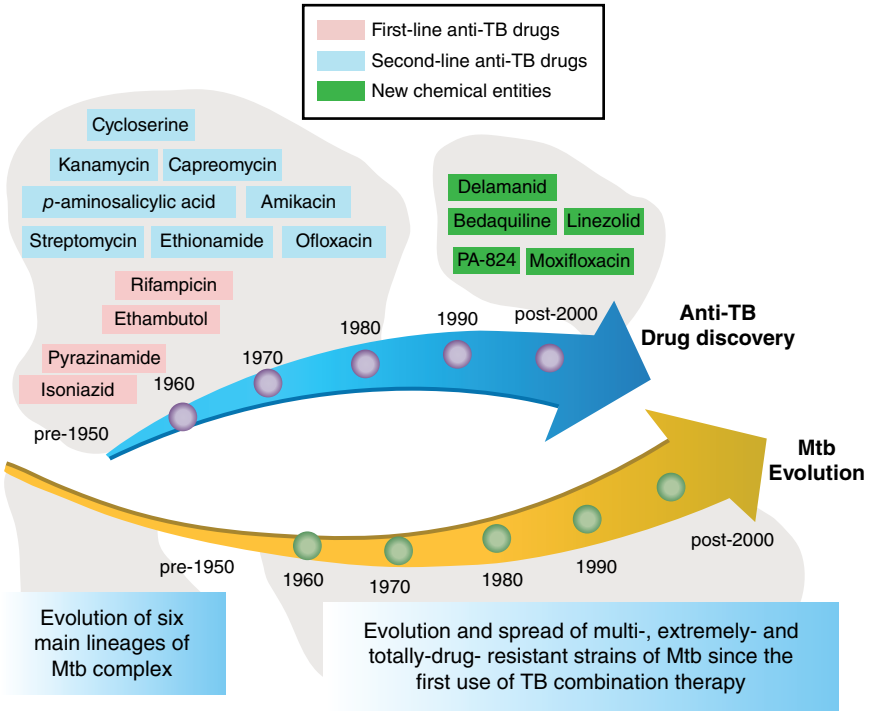


Figure 18.5 *Mtb has the ability to evolve in response to pressure from antibiotics to become drug-resistant. It will continuously adapt and change to the evolutionary pressure and will always be a moving target to new treatment modalities. (See insert for color representation of the figure.)*

18.7.1 Cytokines

The presence of Th1 cytokines at the site of infection, such as IFN-γ and IL-2, is considered protective against Mtb (see § 18.2.5 *Correlates of Protection and Tolerance*). IFN-γ has been administered by different routes in patients harboring drug-sensitive and -resistant mycobacteria [172–175]. IFN-γ administered by the pulmonary route, as an adjunct to standard therapy, led to sputum-negative outcomes faster than for those patients who were administered only the standard therapy [175]. Aerosolized IFN-γ also increased recruitment of lymphocytes and decreased neutrophil counts locally, leading to lower inflammation in the lung. IFN-γ treatment has also been used in MDR-TB patients with good clinical and microbiological improvements, with all patients converting to sputum-negative and a subsequent decrease in bacterial burden [176]. Aerosolized IFN-γ was well-tolerated in these MDR-TB patients, with minor adverse events, such as cough and muscle aches. In another study, aerosolized IFN-γ was administered as adjunctive therapy to six MDR-TB patients after initial treatment failure was observed [177]. Although all patients tolerated inhaled IFN-γ, patients remained sputum-positive after six months of adjunct therapy suggesting that further studies are required to determine aerosolized IFN-γ efficacy in MDR-TB patients.

GM-CSF and IL-2 have also been used to augment the immune response in patients with drug-resistant TB [178, 179]. These two cytokines failed in sputum culture conversion and

consequently no human trials have been conducted in the last 12 years. IL-12 was administered by the subcutaneous (SC) route to one patient for 3 months as an adjuvant to standard therapy [180]. The clinical outcome of the treatment was better; however, larger studies are required to understand the benefit of IL-12 therapy in drug-resistant TB patients.

18.7.2 Vitamin D Therapy

Poor nutritional status of individuals increases the risk of acquiring active TB. Vitamin D has an important immune-modulatory role and its deficiency is known to impair host defense against TB. *In vitro* studies suggest that vitamin D induces IL-1b secretion that accelerates Mtb clearance, possibly due to the presence of the NLRP3/caspase 1 inflammasome in Mtb-infected cells [181, 182]. Calcitriol (metabolically active metabolite of vitamin D; 1,25-dihydroxyvitamin D_3) has shown an anti-mycobacterial effect by generating reactive oxygen and nitrogen species, the transcription of antimicrobial peptides, as well as autophagy [183]. Numerous clinical trials of vitamin D supplementation in TB patients have been conducted with variable outcomes [184–186]. In one of these studies, vitamin D as an adjunct therapy against pulmonary TB induced resolution of inflammatory responses that are usually associated with increased mortality [184].

18.7.3 Re-purposed Drugs

Although we have not achieved much success in developing new anti-TB drugs in the last 50 years, a promising pipeline is emerging through the use of re-purposed drugs to elicit a protective immune response against TB. Non-steroidal anti-inflammatory drugs reduce Mtb burden and also decrease lung lesions in a murine model of TB infection [187, 188]. Efflux-pump inhibitors such as verapamil alone, or as adjunct to anti-TB therapy, could potentially reduce the standard drug dose and shorten the TB treatment regimen in murine models [189, 190]. Shorter treatments were also achieved using phosphodiesterase inhibitors such as cilostazol and sildenafil as an adjunct to standard therapy in a mouse model of TB [191]. The authors of these studies also noted faster Mtb clearance from the lung and reduced tissue damage with the combination therapy. Shorter treatment regimens with re-purposed drugs may significantly improve patient compliance and reduce the emergence of drug-resistant TB strains.

18.7.4 Stem Cell Therapy

Bone marrow-derived autologous mesenchymal stromal cells (MSCs) were administered to MDR-TB patients as a single-dose parenteral infusion. The infusion was well-tolerated and no adverse events were observed at the end of 6 months [192]. The authors of these studies noted that the timing of MSC infusion could play a role in modulating the host immune response, based on whether it was an active or a latent Mtb infection. The proposed mechanism of the MSCs role in TB treatment, as shown in an animal model for sepsis-induced bacterial infection, was the down-regulation of inflammation-related genes and up-regulation of genes involved in phagocytosis [193]. Future human trials will need to include repeated cycles of MSC administration, to ensure no long-term adverse effects. In addition, these trials should focus on the quantity and quality of the immune responses generated in relation to the rate of Mtb clearance, as well as the overall mortality observed in these patients.

The timing of immunotherapy with regards to the state of TB disease is critical in generating a productive immune response that could lead to Mtb clearance. IFN-γ delivery in patients with sepsis, especially when the human leukocyte antigen-antigen D related (HLA-DR) complex was down-regulated on the blood monocytes, was associated with increased survival rate [194]. Administering immunotherapy, especially with a pro-inflammatory cytokine, when there is significant inflammation in the host could lead to a fatal cytokine storm. A cytokine storm is usually accompanied by over-activation of the innate immune system with the release of multiple cytokines. Similarly, IL-17 responses are usually induced at the site of infection during a primary TB infection, and this in turn leads to T cell recruitment and granuloma formation at the infection site [195]. However, during a chronic phase of Mtb infection, a balance between the Th-1 and Th-17 responses is required to control mycobacterial growth and to limit immunopathology due to inflammation. The continued upregulation of IL-17 cytokine could lead to extensive neutrophil recruitment and subsequent tissue damage [196]. Therefore, the application of IL-17 could be a double-edged sword in TB immunotherapy, depending on whether the TB disease is in the primary or the chronic stage of infection [67].

18.8 Conclusion

TB treatment research has been at a standstill for the last 50 years. The existing BCG vaccine was developed nearly 100 years ago, and is still being administered in over 150 countries worldwide despite well-documented variability in its efficacy. In addition, the same TB drugs have been given for more than half a century, and the number of patients diagnosed with MDR-TB has tripled in the last five years [197]. Clinical trials in both the vaccine and therapeutic fields have been fraught with failure, and each new discovery brings about more unanswered questions pertaining to this unique mycobacterium. However, there is hope on the horizon. The TB field can learn from its failures and successes to generate robust immune therapies to control TB. Only 10% of individuals exposed to Mtb develop active infection, which prompts the question: how are current therapeutics and vaccines improving on an already successful human immune system? Perhaps we can follow in the footsteps of other disease fields, harnessing the potential of the patient's own immunity via new immunotherapeutics and vaccines designed to modulate immune responses that are protective during infection. Several immunomodulatory approaches including pre- and post-exposure vaccines, cytokines and their inhibitors, and chemical and biological therapies are being explored to enhance host immunity, either as a stand-alone or an adjunct to existing therapies. These host-directed therapies have been highly successful in the fields of cancer, HIV, and autoimmune disease. Evidence and preliminary work in this field seems to suggest that this may be the only way to compete with this highly resourceful pathogen.

References

[1] Peakman, M. and Dayan, C.M. (2001) Antigen-specific immunotherapy for autoimmune disease: fighting fire with fire? *Immunology*, **104**(4), 361–366.
[2] Rosenberg, S.A., Yang, J.C. and Restifo, N.P. (2004) Cancer immunotherapy: moving beyond current vaccines. *Nature Medicine*, **10**(9), 909–915.

[3] Caspi, R.R. (2008) Immunotherapy of autoimmunity and cancer: the penalty for success. *Nature Reviews Immunology*, **8**(12), 970–976.
[4] Rinaldo, C. (2009) Dendritic cell-based human immunodeficiency virus vaccine. *Journal of Internal Medicine*, **265**(1), 138–158.
[5] Vanham, G. and Van Gulck, E. (2012) Can immunotherapy be useful as a "functional cure" for infection with Human Immunodeficiency Virus-1. *Retrovirology*, **9**(1):72.
[6] Nicas, M., Nazaroff, W.W. and Hubbard, A. (2005) Toward understanding the risk of secondary airborne infection: emission of respirable pathogens. *Journal of Occupational and Environmental Hygiene*, **2**(3), 143–154.
[7] Orme, I.M., Robinson, R.T. and Cooper, A.M. (2015) The balance between protective and pathogenic immune responses in the TB-infected lung. *Nature Immunology*, **16**(1), 57–63.
[8] Ernst, J.D. (2012) The immunological life cycle of tuberculosis. *Nature Reviews Immunology*, **12**(8), 581–591.
[9] Philips, J.A. and Ernst, J.D. (2012) Tuberculosis pathogenesis and immunity. *Annual Review of Pathology: Mechanisms of Disease*, **7**, 353–384.
[10] O'Garra, A., Redford, P.S., McNab, F.W. *et al*. (2013) The immune response in tuberculosis. *Annual Review of Immunology*, **31**, 475–527.
[11] Leemans, J.C., Thepen, T., Weijer, S. *et al*. (2005) Macrophages play a dual role during pulmonary tuberculosis in mice. *Journal of Infectious Diseases*, **191**(1), 65–74.
[12] Ehrt, S. and Schnappinger, D. (2009) Mycobacterial survival strategies in the phagosome: defence against host stresses. *Cellular Microbiology*, **11**(8), 1170–1178.
[13] Behar, S.M., Divangahi, M. and Remold, H.G. (2010) Evasion of innate immunity by Mycobacterium tuberculosis: is death an exit strategy? *Nature Reviews Microbiology*, **8**(9), 668–674.
[14] Divangahi, M., Behar, S.M. and Remold, H. (2013) Dying to live: how the death modality of the infected macrophage affects immunity to tuberculosis, in *The New Paradigm of Immunity to Tuberculosis* (ed. M. divangahi), Springer Science + Business Media, New York, pp. 103–120. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4678885&tool=pmcentrez&rendertype=abstract (accessed 24th May 2016).
[15] Marino, S., Cilfone, N., Mattila, J. *et al*. (2014) Macrophage polarization drives granuloma outcome during Mycobacterium tuberculosis infection. *Journal of Immunology*, **83**(1), 324–338.
[16] Gallegos, A.M., Pamer, E.G. and Glickman, M.S. (2008) Delayed protection by ESAT-6-specific effector CD4+ T cells after airborne M. tuberculosis infection. *Journal of Experimental Medicine*, **205**(10), 2359–2368.
[17] Gutierrez, M.G., Master, S.S., Singh, S.B. *et al.* (2004) Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell*, **119**(6), 753–766.
[18] Martin, C.J., Booty, M.G., Rosebrock, T.R. *et al.* (2012) Efferocytosis is an innate antibacterial mechanism." *Cell Host & Microbe*, **12**(3), 289–300.
[19] Cambier, C., Takaki, K.K., Larson, R.P. *et al.* (2014) Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids. *Nature*, **505**(7482), 218–222.
[20] Cooper, A.M. (2009) Cell-mediated immune responses in tuberculosis. *Annual Review of Immunology*, **27**, 393–422.
[21] Orme, I.M., Andersen, P. and Boom, W.H. (1993) T cell response to Mycobacterium tuberculosis. *Journal of Infectious Diseases*, **167**(6), 1481–1497.
[22] North, R. J. and Jung, Y.J. (2004) Immunity to tuberculosis. *Annual Review of Immunology*, **22**, 599–623.
[23] Sud, D., Bigbee, C., Flynn, J.L. and Kirschner, D.E. (2006) Contribution of CD8+ T cells to control of Mycobacterium tuberculosis infection. *Journal of Immunology*, **176**(7), 4296–4314.
[24] Kumaratne, D.S. (1997) Tuberculosis and immunodeficiency – of mice and men. *Clinical & Experimental Immunology*, **107**(1), 11–14.
[25] Germain, R.N. (2004) An innately interesting decade of research in immunology. *Nature Medicine*, **10**(12), 1307–1320.
[26] Egen, J.G., Rothfuchs, A.G., Feng, C.G. *et al.* (2008) Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. *Immunity*, **28**(2), 271–284.

[27] Maglione, P.J. and Chan, J. (2009) How B cells shape the immune response against Mycobacterium tuberculosis. *European Journal of immunology*, **39**(3), 676–686.
[28] Vordermeier, H., Venkataprasad, N., Harris, D. and Ivanyi, J. (1996) Increase of tuberculous infection in the organs of B cell-deficient mice. *Clinical & Experimental Immunology*, **106**(2), 312–316.
[29] Johnson, C., Cooper, A., Frank, A. *et al*. (1997) Mycobacterium tuberculosis aerogenic rechallenge infections in B cell-deficient mice. *Tubercle and Lung Disease*, **78**(5), 257–261.
[30] Bosio, C.M., Gardner, D. and Elkins, K.L. (2000) Infection of B cell-deficient mice with CDC 1551, a clinical isolate of Mycobacterium tuberculosis: delay in dissemination and development of lung pathology. *Journal of Immunology*, **164**(12), 6417–6425.
[31] Turner, J., Frank, A., Brooks, J. *et al*. (2001) The progression of chronic tuberculosis in the mouse does not require the participation of B lymphocytes or interleukin-4. *Experimental Gerontology*, **36**(3), 537–545.
[32] Maglione, P.J., Xu, J., Casadevall, A. and Chan, J. (2008) Fcγ receptors regulate immune activation and susceptibility during Mycobacterium tuberculosis infection. *Journal of Immunology*, **180**(5), 3329–3338.
[33] Kahnert, A., Höpken, U.E., Stein, M. *et al*. (2007) Mycobacterium tuberculosis triggers formation of lymphoid structure in murine lungs. *Journal of Infectious Diseases*, **195**(1), 46–54.
[34] Moyron-Quiroz, J.E., Rangel-Moreno, J., Kusser, K. *et al*. (2004) Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. *Nature Medicine*, **10**(9), 927–934.
[35] Moyron-Quiroz, J.E., Rangel-Moreno, J., Hartson, L. *et al*. (2006) Persistence and responsiveness of immunologic memory in the absence of secondary lymphoid organs. *Immunity*, **25**(4), 643–654.
[36] Gold, M.C., Cerri, S., Smyk-Pearson, S. *et al*. (2010) Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biology*, **8**(6), e1000407.
[37] Rothchild, A.C., Jayaraman, P., Nunes-Alves, C. and Behar, S.M. (2014) iNKT cell production of GM-CSF controls Mycobacterium tuberculosis. *PLoS Pathogens*, **10**(1), e1003805.
[38] Chen, Z.W. (2013) Multifunctional immune responses of HMBPP-specific V&ggr; 2V&dgr; 2T cells in M. tuberculosis and other infections. *Cellular & Molecular Immunology*, **10**(1), 58–64.
[39] Holden, M., Dubin, M.R. and Diamond, P.H. (1971) Frequency of negative intermediate-strength tuberculin sensitivity in patients with active tuberculosis. *New England Journal of Medicine*, **285**(27), 1506–1509.
[40] Silva Miranda, M., Breiman, A., Allain, S. *et al.* (2012) The tuberculous granuloma: an unsuccessful host defence mechanism providing a safety shelter for the bacteria? *Clinical and Developmental Immunology*, **2012**, 139127.
[41] Davis, J.M., Clay, H., Lewis, J.L. *et al*. (2002) Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. *Immunity*, **17**(6), 693–702.
[42] Davis, J. M. and Ramakrishnan, L. (2008) "The very pulse of the machine": the tuberculous granuloma in motion. *Immunity*, **28**(2), 146–148.
[43] Ramakrishnan, L. (2012) Revisiting the role of the granuloma in tuberculosis. *Nature Reviews Immunology*, **12**(5), 352–366.
[44] Gill, W.P., Harik, N.S., Whiddon, M.R. *et al*. (2009) A replication clock for Mycobacterium tuberculosis. *Nature Medicine*, **15**(2), 211–214.
[45] Fenton, M.J., Vermeulen, M.W., Kim, S. *et al*. (1997) Induction of gamma interferon production in human alveolar macrophages by Mycobacterium tuberculosis. *Infection and Immunity*, **65**(12), 5149–5156.
[46] Wang, J., Wakeham, J., Harkness, R. and Xing, Z. (1999) Macrophages are a significant source of type 1 cytokines during mycobacterial infection. *Journal of Clinical Investigation*, **103**(7), 1023–1029.
[47] Flynn, J.L. and Chan, J. (2001) Immunology of tuberculosis. *Annual Review of Immunology*, **19**(1), 93–129.
[48] Cooper, A.M., Dalton, D.K., Stewart, T.A. *et al.* (1993) Disseminated tuberculosis in interferon gamma gene-disrupted mice. *Journal of Experimental Medicine*, **178**(6), 2243–2247.

[49] Flynn, J.L., Chan, J., Triebold, K.J. *et al*. (1993) An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *Journal of Experimental Medicine*, **178**(6), 2249–2254.
[50] Zhang, M., Lin, Y., Iyer, D.V. *et al*. (1995) T-cell cytokine responses in human infection with Mycobacterium tuberculosis. *Infection and Immunity*, **63**(8), 3231–3234.
[51] Lin, Y., Zhang, M., Hofman, F.M. *et al.* (1996) Absence of a prominent Th2 cytokine response in human tuberculosis. *Infection and Immunity*, **64**(4), 1351–1356.
[52] Ting, L.-M., Kim, A.C., Cattamanchi, A. and Ernst, J.D. (1999) Mycobacterium tuberculosis inhibits IFN-γ transcriptional responses without inhibiting activation of STAT1. *Journal of Immunology*, **163**(7), 3898–3906.
[53] Flynn, J.L., Goldstein, M.M., Chan, J. *et al*. (1995) Tumor necrosis factor-α is required in the protective immune response against Mycobacterium tuberculosis in mice. *Immunity*, **2**(6), 561–572.
[54] Bean, A.G., Roach, D.R., Briscoe, H. *et al*. (1999) Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. *Journal of Immunology*, **162**(6), 3504–3511.
[55] Mohan, V.P., Scanga, C.A., Yu, K. *et al*. (2001) Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology. *Infection and Immunity*, **69**(3), 1847–1855.
[56] Nadkarni, S., Mauri, C. and Ehrenstein, M.R. (2007) Anti–TNF-α therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-β. *Journal of Experimental Medicine*, **204**(1), 33–39.
[57] Clay, H., Volkman, H.E. and Ramakrishnan, L. (2008) Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. *Immunity*, **29**(2), 283–294.
[58] Wallis, R.S. (2008) Tumour necrosis factor antagonists: structure, function, and tuberculosis risks. *The Lancet Infectious Diseases*, **8**(10), 601–611.
[59] Rook, G., Taverne, J., Leveton, C. and Steele, J. (1987) The role of gamma-interferon, vitamin D3 metabolites and tumour necrosis factor in the pathogenesis of tuberculosis. *Immunology*, **62**(2), 229–234.
[60] Rook, G. (1990) Mycobacteria, cytokines and antibiotics. *Pathologie-Biologie*, **38**(4), 276–280.
[61] Moreira, A., Tsenova-Berkova, L., Wang, J. *et al*. (1997) Effect of cytokine modulation by thalidomide on the granulomatous response in murine tuberculosis. *Tubercle and Lung Disease*, **78**(1), 47–55.
[62] Gong, J.-H., Zhang, M., Modlin, R.L. *et al*. (1996) Interleukin-10 downregulates Mycobacterium tuberculosis-induced Th1 responses and CTLA-4 expression. *Infection and Immunity*, **64**(3), 913–918.
[63] Rojas, R.E., Balaji, K.N., Subramanian, A. and Boom, W.H. (1999) Regulation of human CD4+ αβ T-cell-receptor-positive (TCR+) and γδ TCR+ T-cell responses to Mycobacterium tuberculosis by interleukin-10 and transforming growth factor β. *Infection and Immunity*, **67**(12), 6461–6472.
[64] Ding, A., Nathan, C.F., Graycar, J. *et al.* (1990) Macrophage deactivating factor and transforming growth factors-beta 1-beta 2 and-beta 3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-gamma. *Journal of Immunology*, **145**(3), 940–944.
[65] Hirsch, C.S., Ellner, J.J., Blinkhorn, R. and Toossi, Z. (1997) In vitro restoration of T cell responses in tuberculosis and augmentation of monocyte effector function against Mycobacterium tuberculosis by natural inhibitors of transforming growth factor β. *Proceedings of the National Academy of Sciences of the United States of America*, **94**(8), 3926–3931.
[66] McNab, F.W., Berry, M.P., Graham, C.M. *et al*. (2011) Programmed death ligand 1 is overexpressed by neutrophils in the blood of patients with active tuberculosis. *European Journal of Immunology*, **41**(7), 1941–1947.
[67] Uhlin, M., Andersson, J., Zumla, A. and Maeurer, M. (2012) Adjunct immunotherapies for tuberculosis. *Journal of Infectious Diseases*, **205**(Supplement 2), S325–S334.
[68] Young, D. (2009) Animal models of tuberculosis. *European Journal of Immunology*, **39**(8), 2011–2014.

[69] Orme, I. (2003) The mouse as a useful model of tuberculosis. *Tuberculosis (Edinburgh)*, **83**(1), 112–115.
[70] Via, L.E., Lin, P.L., Ray, S.M. *et al*. (2008) Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. *Infection and Immunity*, **76**(6), 2333–2340.
[71] Harper, J., Skerry, C., Davis, S.L. *et al*. (2011) Mouse model of necrotic tuberculosis granulomas develops hypoxic lesions. *Journal of Infectious Diseases*, **205**(4), 595–602.
[72] Driver, E.R., Ryan, G.J., Hoff, D.R. *et al*. (2012) Evaluation of a mouse model of necrotic granuloma formation using C3HeB/FeJ mice for testing of drugs against Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*, **56**(6), 3181–3195.
[73] Calderon, V.E., Valbuena, G., Goez, Y. *et al*. (2013) A humanized mouse model of tuberculosis. *PLoS One*, **8**(5), e63331.
[74] Apt, A. and Kramnik, I. (2009) Man and mouse TB: contradictions and solutions. *Tuberculosis (Edinburgh)*, **89**(3), 195–198.
[75] Capuano, S.V., Croix, D.A., Pawar, S. *et al*. (2003) Experimental Mycobacterium tuberculosis infection of cynomolgus macaques closely resembles the various manifestations of human M. tuberculosis infection. *Infection and Immunity*, **71**(10), 5831–5844.
[76] Lin, P.L., Rodgers, M., Smith, L.K. *et al*. (2009) Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. *Infection and Immunity*, **77**(10), 4631–4642.
[77] Darrah, P.A., Bolton, D.L., Lackner, A.A. *et al.* (2014) Aerosol Vaccination with AERAS-402 Elicits Robust Cellular Immune Responses in the Lungs of Rhesus Macaques but Fails To Protect against High-Dose Mycobacterium tuberculosis Challenge. *Journal of Immunology*, **193**(4), 1799–1811.
[78] Satti, I., Meyer, J., Harris, S.A. *et al*. (2014) Safety and immunogenicity of a candidate tuberculosis vaccine MVA85A delivered by aerosol in BCG-vaccinated healthy adults: a phase 1, double-blind, randomised controlled trial. *The Lancet Infectious Diseases*, **14**(10), 939–946.
[79] Calmette, A. and Plotz, H. (1929) Protective inoculation against tuberculosis with BCG. *American Review of Tuberculosis*, **19**, 567–572.
[80] World Health Organization (2004) BCG vaccine. WHO position paper. *Weekly Epidemiological Record*, **79**(4), 27–38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14768305 (accessed 24th May 2016).
[81] Tullius, M.V. and Horwitz, M.A. (2011) New Generation BCG Vaccines, in *Replicating Vaccines* (eds P.R. Dormitzer, C.W. Mandl and R. Rappuilo), Springer, Basel, pp. 119–169. Available from: http://www.springerlink.com/index/10.1007/978-3-0346-0277-8 (accessed 24th May 2016)
[82] Behr, M., Wilson, M., Gill, W. *et al*. (1999) Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science*, **284**(5419), 1520–1523.
[83] Sakula, A. (1983) BCG: who were Calmette and Guerin? *Thorax*, **38**(11), 806–812.
[84] Rosenthal, S.R., McEnery, J.T. and Raisys, N. (1968) Aerogenic BCG vaccination against tuberculosis in animal and human subjects. *Journal of Asthma*, **5**(4), 309–323.
[85] Palendira, U., Bean, A.G., Feng, C.G. and Britton, W.J. (2002) Lymphocyte recruitment and protective efficacy against pulmonary mycobacterial infection are independent of the route of prior Mycobacterium bovis BCG immunization. *Infection and Immunity*, **70**(3), 1410–1416.
[86] Chen, L., Wang, J., Zganiacz, A. and Xing, Z. (2003) Single Intranasal Mucosal Mycobacterium bovis BCG Vaccination Confers Improved Protection Compared to Subcutaneous Vaccination against Pulmonary Tuberculosis. *Infection and Immunity*, **72**(1), 238–246.
[87] Mittrucker, H.W., Steinhoff, U., Kohler, A. *et al*. (2007) Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. *Proceedings of the National Acadademy of Sciences of the United States of America*, **104**(30), 12434–12439.
[88] Garcia-Contreras, L., Wong, Y.L., Muttil, P. *et a*l. (2008) Immunization by a bacterial aerosol. *Proceedings of the National Academy of Sciences of the United States of America*, **105**(12), 4656–4660.
[89] Aguilo, N., Toledo, A.M., Lopez-Roman, E.M. *et al*. (2014) Pulmonary Mycobacterium bovis BCG vaccination confers dose-dependent superior protection compared to that of subcutaneous vaccination. *Clinical and Vaccine Immunology*, **21**(4), 594–597.

[90] Derrick, S.C., Kolibab, K., Yang, A. and Morris, S.L. (2014) Intranasal Administration of Mycobacterium bovis BCG Induces Superior Protection against Aerosol Infection with Mycobacterium tuberculosis in Mice. *Clinical and Vaccine Immunology*, **21**(10), 1443–1451.

[91] Ajdary, S., Dobakhti, F., Taghikhani, M. *et al*. (2007) Oral administration of BCG encapsulated in alginate microspheres induces strong Th1 response in BALB/c mice. *Vaccine*, **25**(23), 4595–4601.

[92] Aldwell, F., Cross, M., Fitzpatrick, C. *et al*. (2006) Oral delivery of lipid-encapsulated Mycobacterium bovis BCG extends survival of the bacillus in vivo and induces a long-term protective immune response against tuberculosis. *Vaccine*, **24**(12), 2071–2078.

[93] WHO (1979) Trial of BCG vaccines in south India for tuberculosis prevention. First report. *Bulletin of the World Health Organization*: Tuberculosis Prevention Trial.

[94] Hart, P.A. and Sutherland, I. (1977) BCG and vole bacillus vaccines in the prevention of tuberculosis in adolescence and early adult life. *BMJ*, **2**(6082), 293–295.

[95] Ten Dam, H. and Hitze, K. (1980) Does BCG vaccination protect the newborn and young infants? *Bulletin of the World Health Organization*, **58**(1), 37–41.

[96] Colditz, G.A., Brewer, T.F., Berkey, C.S. *et al*. (1994) Efficacy of BCG vaccine in the prevention of tuberculosis: meta-analysis of the published literature. *JAMA*, **271**(9), 698–702.

[97] Daniel, T.M. and Janicki, B.W. (1978) Mycobacterial antigens: a review of their isolation, chemistry, and immunological properties. *Microbiological Reviews*, **42**(1), 84–113.

[98] Rogall, T., Wolters, J., Flohr, T. and Bottger, E.C. (1990) Towards a phylogeny and definition of species at the molecular level within the genus Mycobacterium. *International Journal of Systematic Bacteriology*, **40**(4), 323–330.

[99] Zelazny, A.M., Calhoun, L.B., Li, L. *et al.* (2005) Identification of Mycobacterium species by secA1 sequences. *Journal of Clinical Microbiology*, **43**(3), 1051–1058.

[100] Kamala, T., Paramasivan, C., Herbert, D. *et al*. (1994) Isolation and identification of environmental mycobacteria in the Mycobacterium bovis BCG trial area of South India. *Applied and Environmental Microbiology*, **60**(6), 2180–2183.

[101] Brandt, L., Cunha, J.F., Olsen, A.W. *et al*. (2002) Failure of the Mycobacterium bovis BCG vaccine: Some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infection and Immunity*, **70**(2), 672–678.

[102] Demangel, C., Garnier, T., Rosenkrands, I. and Cole, S.T. (2005) Differential effects of prior exposure to environmental mycobacteria on vaccination with Mycobacterium bovis BCG or a recombinant BCG strain expressing RD1 antigens. *Infection and Immunity*, **73**(4), 2190–2196.

[103] Flaherty, D.K., Vesosky, B., Beamer, G.L. *et al.* (2006) Exposure to Mycobacterium avium can modulate established immunity against Mycobacterium tuberculosis infection generated by Mycobacterium bovis BCG vaccination. *Journal of Leukocyte Biology*, **80**(6), 1262–1271.

[104] Young, S.L., Slobbe, L., Wilson, R. *et al*. (2007) Environmental strains of Mycobacterium avium interfere with immune responses associated with Mycobacterium bovis BCG vaccination. *Infection and Immunity*, **75**(6), 2833–2840.

[105] Poyntz, H.C., Stylianou, E., Griffiths, K.L. *et al*. (2014) Non-tuberculous mycobacteria have diverse effects on BCG efficacy against Mycobacterium tuberculosis. *Tuberculosis (Edinburgh)*, **94**(3), 226–237.

[106] Palmer, C. and Long, M.W. (1966) Effects of infection with atypical mycobacteria on BCG vaccination and tuberculosis. *American Review of Respiratory Disease*, **94**(4), 553–568.

[107] Andersen, P. and Doherty, M. (2005) The success and failure of BCG – implications for a novel tuberculosis vaccine. *Nature Reviews Microbiology*, **3**(8), 656–662.

[108] Black, G.F., Weir, R.E., Floyd, S. *et al*. (2002) BCG-induced increase in interferon-gamma response to mycobacterial antigens and efficacy of BCG vaccination in Malawi and the UK: two randomised controlled studies. *The Lancet*, **359**(9315), 1393–1401.

[109] McShane, H. (2014) Editorial commentary: understanding BCG is the key to improving it. *Clinical Infectious Diseases*, **58**(4), 481–482.

[110] Tsuji, N.M. and Kosaka, A. (2008) Oral tolerance: intestinal homeostasis and antigen-specific regulatory T cells. *Trends in Immunology*, **29**(11), 532–540.
[111] Price, D.N., Kusewitt, D.F., Lino, C.A. *et al*. (2016) Oral tolerance to environmental mycobacteria interferes with intradermal, but not pulmonary, immunization against tuberculosis. *PLoS Pathogens*, **12**(5), e1005614.
[112] Behr, M.A. (2002) BCG – different strains, different vaccines? *The Lancet Infectious Diseases*, **2**(2), 86–92.
[113] McShane, H., Jacobs, W.R., Fine, P.E. *et al*. (2012) BCG: myths, realities, and the need for alternative vaccine strategies. *Tuberculosis (Edinburgh)*, **92**(3), 283–288.
[114] Gagneux, S., DeRiemer, K., Van, T. *et al*. (2006) Variable host–pathogen compatibility in Mycobacterium tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America*, **103**(8), 2869–2873.
[115] Portevin, D., Gagneux, S., Comas, I. and Young, D. (2011) Human macrophage responses to clinical isolates from the Mycobacterium tuberculosis complex discriminate between ancient and modern lineages. *PLoS Pathogens*, **7**(3), e1001307.
[116] WHO (2012) Soil transmitted helminthiases: Eliminating soil- transmitted helminthiases as a public health problem in children. *Progress Report* 2001–2010 and *Strategic Plan* 2011–2020.
[117] Rook, G.A., Dheda, K. and Zumla, A. (2005) Immune responses to tuberculosis in developing countries: implications for new vaccines. *Nature Reviews Immunology*, **5**(8), 661–667.
[118] Lycke, N. (2012) Recent progress in mucosal vaccine development: potential and limitations. *Nature Reviews Immunology*, **12**(8), 592–605.
[119] Nascimento, I. and Leite, L. (2012) Recombinant vaccines and the development of new vaccine strategies. *Brazilian Journal of Medical and Biological Research*, **45**(12), 1102–1111.
[120] Hoft, D.F., Leonardi, C., Milligan, T. *et al*. (1999) Clinical reactogenicity of intradermal bacille Calmette-Guerin vaccination. *Clinical Infectious Diseases*, **28**(4), 785–790.
[121] Orme, I. (1988) Induction of nonspecific acquired resistance and delayed-type hypersensitivity, but not specific acquired resistance in mice inoculated with killed mycobacterial vaccines. *Infection and Immunity*, **56**(12), 3310–3312.
[122] Daugelat, S., Ladel, C.H. and Kaufmann, S. (1995) Influence of mouse strain and vaccine viability on T-cell responses induced by Mycobacterium bovis bacillus Calmette-Guérin. *Infection and Immunity*, **63**(5), 2033–2040.
[123] Martin, C., Williams, A., Hernandez-Pando, R. *et al*. (2006) The live Mycobacterium tuberculosis phoP mutant strain is more attenuated than BCG and confers protective immunity against tuberculosis in mice and guinea pigs. *Vaccine*, **24**(17), 3408–3419.
[124] Conradt, P., Hess, J. and Kaufmann, S.H. (1999) Cytolytic T-cell responses to human dendritic cells and macrophages infected with Mycobacterium bovis BCG and recombinant BCG secreting listeriolysin. *Microbes and Infection*, **1**(10), 753–764.
[125] Detmer, A. and Glenting, J. (2006) Live bacterial vaccines – a review and identification of potential hazards. *Microbial Cell Factories*, **5**:23.
[126] Walker, K., Brennan, M., Ho, M. *et al*. (2010) The second Geneva Consensus: Recommendations for novel live TB vaccines. *Vaccine*, **28**(11), 2259–2270.
[127] Vuola, J.M., Ristola, M.A., Cole, B. *et al*. (2003) Immunogenicity of an inactivated mycobacterial vaccine for the prevention of HIV-associated tuberculosis: a randomized, controlled trial. *AIDS*, **17**(16), 2351–2355.
[128] Von Reyn, C., Bakari, M., Arbeit, R. *et al*. (2012) New vaccines for the prevention of tuberculosis in human immunodeficiency virus infection [Perspectives]. *International Journal of Tuberculosis and Lung Disease*, **16**(6), 718–723.
[129] Waddell, R.D., Chintu, C., Lein, A.D. *et al*. (2000) Safety and immunogenicity of a five-dose series of inactivated Mycobacterium vaccae vaccination for the prevention of HIV-associated tuberculosis. *Clinical Infectious Diseases*, **30**(Supplement 3), S309–S315.

[130] Von Reyn, C.F., Mtei, L., Arbeit, R.D. *et al*. (2010) Prevention of tuberculosis in Bacille Calmette-Guerin-primed, HIV-infected adults boosted with an inactivated whole-cell mycobacterial vaccine. *AIDS*, **24**(5), 675–685.

[131] McShane, H. (2011) Tuberculosis vaccines: beyond bacille Calmette-Guerin. *Philosophical Transactions of the Royal Society London B: Biological Sciences*, **366**(1579), 2782–2789.

[132] Tameris, M.D., Hatherill, M., Landry, B.S. *et al.* (2013) Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *The Lancet*, **381**(9871), 1021–1028.

[133] Goonetilleke, N.P., McShane, H., Hannan, C.M. *et al*. (2003) Enhanced Immunogenicity and Protective Efficacy Against Mycobacterium tuberculosis of Bacille Calmette-Guerin vaccine Using Mucosal Administration and Boosting with a Recombinant Modified Vaccinia Virus Ankara. *Journal of Immunology*, **171**(3), 1602–1609.

[134] Williams, A., Goonetilleke, N.P., McShane, H. *et al*. (2005) Boosting with poxviruses enhances Mycobacterium bovis BCG efficacy against tuberculosis in guinea pigs. *Infection and Immunoty*, **73**(6), 3814–3816.

[135] Verreck, F.A., Vervenne, R.A., Kondova, I. *et al*. (2009) MVA.85A boosting of BCG and an attenuated, phoP deficient M. tuberculosis vaccine both show protective efficacy against tuberculosis in rhesus macaques. *PLoS One*, **4**(4), e5264.

[136] Vordermeier, H.M., Villarreal-Ramos, B., Cockle, P.J. *et al*. (2009) Viral booster vaccines improve Mycobacterium bovis BCG-induced protection against bovine tuberculosis. *Infection and Immunity*, **77**(8), 3364–3373.

[137] Abel, B., Tameris, M., Mansoor, N. *et al*. (2010) The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults. *American Journal of Respiratory and Critical Care Medicine*, **181**(12), 1407–1417.

[138] Buchbinder, S.P., Mehrotra, D.V., Duerr, A. and Magg, R. (2008) Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *The Lancet*, **372**(9653), 1881–1893.

[139] Gray, G.E., Allen, M., Moodie, Z. *et al*. (2011) Safety and efficacy of the HVTN 503/Phambili Study of a clade-B-based HIV-1 vaccine in South Africa: a double-blind, randomised, placebo-controlled test-of-concept phase 2b study. *The Lancet Infectious Diseases*, **11**(7), 507–515.

[140] Hammer, S.M., Sobieszczyk, M.E., Janes, H. *et al*. (2013) Efficacy trial of a DNA/rAd5 HIV-1 preventive vaccine. *New England Journal of Medicine*, **369**(22), 2083–2092.

[141] Andersen, P. (2007) Vaccine strategies against latent tuberculosis infection. *Trends in Microbiology*, **15**(1), 7–13.

[142] Gallant, C.J., Cobat, A., Simkin, L. *et al*. (2010) Impact of age and sex on mycobacterial immunity in an area of high tuberculosis incidence. *International Journal of Tuberculosis and Lung Disease*, **14**(8), 952–959.

[143] Ottenhoff, T.H. and Kaufmann, S.H. (2012) Vaccines against tuberculosis: where are we and where do we need to go? *PLoS Pathogens*, **8**(5), e1002607.

[144] Von Eschen, K., Morrison, R., Braun, M. *et al*. (2014) The candidate tuberculosis vaccine Mtb72F/AS02A: Tolerability and immunogenicity in humans. *Human Vaccines*, **5**(7), 475–482.

[145] Aagaard, C., Hoang, T., Dietrich, J. *et al*. (2011) A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nature Medicine*, **17**(2), 189–194.

[146] Pai, M., Zwerling, A. and Menzies, D. (2008) Systematic review: T-cell–based assays for the diagnosis of latent tuberculosis infection: an update. *Annals of Internal Medicine*, **149**(3), 177–184.

[147] Demissie, A., Leyten, E.M., Abebe, M. *et al*. (2006) Recognition of stage-specific mycobacterial antigens differentiates between acute and latent infections with Mycobacterium tuberculosis. *Clinical and Vaccine Immunology*, **13**(2), 179–186.

[148] Govender, L., Abel, B., Hughes, E.J. *et al*. (2010) Higher human CD4 T cell response to novel Mycobacterium tuberculosis latency associated antigens Rv2660 and Rv2659 in latent infection compared with tuberculosis disease. *Vaccine*, **29**(1), 51–57.

[149] Lin, M.Y., Geluk, A., Smith, S.G. *et al*. (2007) Lack of immune responses to Mycobacterium tuberculosis DosR regulon proteins following Mycobacterium bovis BCG vaccination. *Infection and Immunity*, **75**(7), 3523–3530.

[150] Reece, S.T., Nasser-Eddine, A., Dietrich, J. *et al*. (2011) Improved long-term protection against Mycobacterium tuberculosis Beijing/W in mice after intra-dermal inoculation of recombinant BCG expressing latency associated antigens. *Vaccine*, **29**(47), 8740–8744.

[151] Olsen, A.W., Williams, A., Okkels, L.M. *et al*. (2004) Protective effect of a tuberculosis subunit vaccine based on a fusion of antigen 85B and ESAT-6 in the aerosol guinea pig model. *Infection and Immunity*, **72**(10), 6148–6150.

[152] Lin, P.L., Dietrich, J., Tan, E. *et al*. (2012) The multistage vaccine H56 boosts the effects of BCG to protect cynomolgus macaques against active tuberculosis and reactivation of latent Mycobacterium tuberculosis infection. *Journal of Clinical Investigation*, **122**(1), 303–314.

[153] Hoang, T., Aagaard, C., Dietrich, J. *et al*. (2013) ESAT-6 (EsxA) and TB10. 4 (EsxH) based vaccines for pre-and post-exposure tuberculosis vaccination. *PLoS One*, **8**(12), e80579.

[154] Ota, M.O., Odutola, A.A., Owiafe, P.K. *et al*. (2011) Immunogenicity of the tuberculosis vaccine MVA85A is reduced by coadministration with EPI vaccines in a randomized controlled trial in Gambian infants. *Science Translational Medicine*, **3**(88), 88ra56–88ra56.

[155] Brennan, M.J., Stone, M.R. and Evans, T. (2012) A rational vaccine pipeline for tuberculosis. *International Journal of Tuberculosis and Lung Disease*, **16**(12), 1566–1573.

[156] Benyus, J.M. (1997). *Biomimicry*, William Morrow, New York.

[157] Jeyanathan, M., Heriazon, A. and Xing, Z. (2010) Airway luminal T cells: a newcomer on the stage of TB vaccination strategies. *Trends in Immunology*, **31**(7), 247–252.

[158] Muttil, P., Price, D. and McBride, A. (2011) Pulmonary immunization for TB with live cell-based vaccines: the importance of the delivery route. *Therapeutic Delivery*, **2**(12), 1519–1522. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22833980 (accessed 24th May 2016).

[159] Giri, P.K., Verma, I. and Khuller, G.K. (2006) Protective efficacy of intranasal vaccination with Mycobacterium bovis BCG against airway Mycobacterium tuberculosis challenge in mice. *Journal of Infection*, **53**(5), 350–356.

[160] Lu, D. and Hickey, A. (2007) Pulmonary vaccine delivery. *Expert Review of Vaccines*, **6**, 213–226.

[161] Hokey, D.A. and Misra, A. (2011) Aerosol vaccines for tuberculosis: a fine line between protection and pathology. *Tuberculosis (Edinburgh)*, **91**(1), 82–85.

[162] Laddy, D.J., Graves, A.J., Misra, A. and Hokey, D.A. (2014) Inhalable vaccines for TB. *The Art & Science of Tuberculosis Vaccine Development*, 2nd edn, Oxford University Press, pp. 1027–1054.

[163] Horvath, C.N., Shaler, C.R., Jeyanathan, M. *et al*. (2012) Mechanisms of delayed anti-tuberculosis protection in the lung of parenteral BCG-vaccinated hosts: a critical role of airway luminal T cells. *Mucosal Immunology*, **5**(4), 420–431.

[164] Shaler, C.R., Horvath, C., Lai, R. and Xing, Z. (2012) Understanding delayed T-cell priming, lung recruitment, and airway luminal T-cell responses in host defense against pulmonary tuberculosis. *Clinical and Developmental Immunology*, **2012**, 628293.

[165] Mora, J.R., Bono, M.R., Manjunath, N. *et al*. (2003) Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature*, **424**(6944), 88–93.

[166] Iwata, M., Hirakiyama, A., Eshima, Y. *et al*. (2004) Retinoic acid imprints gut-homing specificity on T cells. *Immunity*, **21**(4), 527–538.

[167] Holmgren, J. and Czerkinsky, C. (2005) Mucosal immunity and vaccines. *Nature Medicine*, **11**, S45–S53.

[168] Muttil, P., Prego, C., Garcia-Contreras, L. *et al*. (2010) Immunization of guinea pigs with novel hepatitis B antigen as nanoparticle aggregate powders administered by the pulmonary route. *AAPS Journal*, **12**(3), 330–337.

[169] Wang, C., Muttil, P., Lu, D. *et al*. (2009) Screening for potential adjuvants administered by the pulmonary route for tuberculosis vaccines. *AAPS Journal*, **11**(1), 139–147.

[170] Garner, F.B., Meyer, C.A., White, D.S. and Lipton, A. (1975) Aerosol BCG treatment of carcinoma metastatic to the lung: A phase I study. *Cancer*, **35**, 1088–1094.

[171] Xing, Z. and Lichty, B.D. (2006) Use of recombinant virus-vectored tuberculosis vaccines for respiratory mucosal immunization. *Tuberculosis (Edinburgh)*, **86**(3–4), 211–217.

[172] Giosue, S., Casarini, M., Alemanno, L. *et al*. (1998) Effects of aerosolized interferon-α in patients with pulmonary tuberculosis. *American Journal of Respiratory and Critical Care Medicine*, **158**(4), 1156–1162.

[173] Giosue, S., Casarini, M., Ameglio, F. *et al*. (2000) Aerosolized interferon-alpha treatment in patients with multi-drug-resistant pulmonary tuberculosis. *European Cytokine Network*, **11**(1), 99–104.

[174] Suárez-Méndez, R., García-García, I., Fernández-Olivera, N. *et al*. (2004) Adjuvant interferon gamma in patients with drug-resistant pulmonary tuberculosis: a pilot study. *BMC Infectious Diseases*, **4**(1):44.

[175] Dawson, R., Condos, R., Tse, D. *et al.* (2009) Immunomodulation with recombinant interferon-gamma1b in pulmonary tuberculosis. *PLoS One*, **4**(9), e6984.

[176] Condos, R., Rom, W.N. and Schluger, N.W. (1997) Treatment of multidrug-resistant pulmonary tuberculosis with interferon-γ via aerosol. *The Lancet*, **349**(9064), 1513–1515.

[177] Koh, W.-J., Kwon, O.J., Suh, G.Y. *et al*. (2004) Six-month therapy with aerosolized interferon-γ for refractory multidrug-resistant pulmonary tuberculosis. *Journal of Korean Medical Science*, **19**(2), 167–171.

[178] Johnson, J.L., Ssekasanvu, E., Okwera, A. *et al*. (2003) Randomized trial of adjunctive interleukin-2 in adults with pulmonary tuberculosis. *American Journal of Respiratory and Critical Care Medicine*, **168**(2), 185–191.

[179] Pedral-Sampaio, D.B., Netto, E.M., Brites, C. *et al*. (2003) Use of Rhu-GM-CSF in pulmonary tuberculosis patients: results of a randomized clinical trial. *Brazilian Journal of Infectious Diseases*, **7**(4), 245–252.

[180] Greinert, U., Ernst, M., Schlaak, M. and Entzian, P. (2001) Interleukin-12 as successful adjuvant in tuberculosis treatment. *European Respiratory Journal*, **17**(5), 1049–1051.

[181] Verway, M., Bouttier, M., Wang, T.-T. *et al*. (2013) Vitamin D induces interleukin-1β expression: paracrine macrophage epithelial signaling controls M. tuberculosis infection. *PLoS Pathogens*, **9**(6), e1003407.

[182] Martineau, A.R., Wilkinson, R.J., Wilkinson, K.A. *et al*. (2007) A single dose of vitamin D enhances immunity to mycobacteria. *American Journal of Respiratory and Critical Care Medicine*, **176**(2), 208–213. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17463418 [cited 2016 May 11].

[183] Liu, P.T., Schenk, M., Walker, V.P. *et al.* (2009) Convergence of IL-1β and VDR activation pathways in human TLR2/1-induced antimicrobial responses. *PLoS One*, **4**(6), e5810. Available from: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0005810 [cited 2016 May 11].

[184] Coussens, A.K., Wilkinson, R.J., Hanifa, Y. *et al.* (2012) Vitamin D accelerates resolution of inflammatory responses during tuberculosis treatment. *Proceedings of the National Academy of Sciences of the United States of America*, **109**(38), 15449–15454. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3458393&tool=pmcentrez&rendertype=abstract [cited 2016 May 1].

[185] Wejse, C., Gomes, V.F., Rabna, P. *et al.* (2009) Vitamin D as supplementary treatment for tuberculosis: a double-blind, randomized, placebo-controlled trial. *American Journal of*

Respiratory and Critical Care Medicine, **179**(9), 843–850. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19179490 [cited 2016 April 19].

[186] Martineau, A.R., Timms, P.M., Bothamley, G.H. *et al*. (2011) High-dose vitamin D(3) during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. *The Lancet (London, England)*, **377**(9761), 242–250. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4176755&tool=pmcentrez&rendertype=abstract [cited 2016 March 18].

[187] Vilaplana, C., Marzo, E., Tapia, G. *et al.* (2013) Ibuprofen therapy resulted in significantly decreased tissue bacillary loads and increased survival in a new murine experimental model of active tuberculosis. *Journal of Infectious Diseases*, **208**(2), 199–202.

[188] Guzman, J.D., Evangelopoulos, D., Gupta, A. *et al*. (2013) Antitubercular specific activity of ibuprofen and the other 2-arylpropanoic acids using the HT-SPOTi whole-cell phenotypic assay. *BMJ Open*, **3**(6), e002672. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3693423&tool=pmcentrez&rendertype=abstract [cited 2016 May 11].

[189] Gupta, S., Tyagi, S., Almeida, D.V. *et al.* (2013) Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an efflux inhibitor. *American Journal of Respiratory and Critical Care Medicine*, **188**(5), 600–607.

[190] Gupta, S., Cohen, K.A., Winglee, K. *et al*. (2014) Efflux inhibition with verapamil potentiates bedaquiline in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*, **58**(1), 574–576.

[191] Maiga, M., Ammerman, N.C., Maiga, M.C. *et al*. (2013) Adjuvant host-directed therapy with type 3 and 5 phosphodiesterase inhibitors, but not type 4, shortens the duration of tuberculosis treatment. *Journal of Infectious Diseases*, **208**(3), 512–519.

[192] Skrahin, A., Ahmed, R.K., Ferrara, G. *et al*. (2014) Autologous mesenchymal stromal cell infusion as adjunct treatment in patients with multidrug and extensively drug-resistant tuberculosis: an open-label phase 1 safety trial. *The Lancet Respiratory Medicine*, **2**(2), 108–122.

[193] Krasnodembskaya, A., Song, Y., Fang, X. *et al*. (2010) Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells*, **28**(12), 2229–2238.

[194] Döcke, W.-D., Randow, F., Syrbe, U. *et al*. (1997) Monocyte deactivation in septic patients: restoration by IFN-γ treatment. *Nature Medicine*, **3**(6), 678–681.

[195] Umemura, M., Yahagi, A., Hamada, S. *et al*. (2007) IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection. *Journal of Immunology*, **178**(6), 3786–3796.

[196] Cruz, A., Fraga, A.G., Fountain, J.J. *et al*. (2010) Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with Mycobacterium tuberculosis. *Journal of Experimental Medicine*, **207**(8), 1609–1616.

[197] WHO (2014) *Global Tuberculosis Report* 2014. Available from http://apps.who.int/iris/bitstream/10665/137094/1/9789241564809_eng.pdf (accessed 24th May 2016).

Section 6
Clinical Perspective

19

Clinical and Public Health Perspectives

Ruvandhi R. Nathavitharana[1] *and Edward A. Nardell*[2]

[1] *Division of Infectious Diseases, Beth Israel Deaconess Medical Center, Boston, MA 02215*
[2] *Division of Global Health Equity, Brigham and Women's Hospital, Boston, MA 02115*

19.1 Introduction

Most of this book covers the development and testing of new drug delivery systems for tuberculosis (TB), especially the inhaled route. However, long before drugs get into patients, there are practical, clinical and public health considerations that drug developers should understand. For example, developers should appreciate that TB, especially in immunosuppressed persons or patients with other lung diseases, is a systemic infection. Therefore new drug delivery systems that target the lungs may ultimately fail if either systemic therapeutic levels are not achieved through pulmonary delivery, or if not accompanied by adequate systemic therapy. Likewise, from a public health perspective, there is broad agreement that the treatment of multi-drug resistant (MDR) TB must be fully supervized but that reliable (non-family member) supervision more than once a day is logistically difficult and expensive. Thus, new drug delivery systems that require two or three times per day dosing may have acceptable pharmacokinetics and potentially work in clinical trials, but fail in practice due to difficulty with supervision. It goes without saying that managing drug toxicity for 6 to 24 months of treatment is a major clinical and public health challenge. Many current drugs, including isoniazid, are more toxic than would generally be acceptable for other disease treatments by today's standards, but there are currently few alternatives for TB.

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

Ideally, new delivery systems should be less toxic than current systems and regimens, as this is an instance where equivalence is really not acceptable.

19.2 Background

Historically, since the discovery of the first anti-tuberculosis drugs, streptomycin in 1944, *p*-aminosalicylate sodium (PAS) in 1946, and isoniazid (INH) in 1952, TB treatment has focused on oral and injectable drugs only. By 1955, the awareness of the emergence of drug-resistant strains led to consensus regarding the need for combination therapy, which initially consisted of a 2-year course of streptomycin, PAS and INH. With the advent of rifampin (RIF) in 1959, the therapeutic course was decreased to 9 months. After ethambutol (EMB) and pyrazinamide (PZA) were discovered in the early 1960s, the treatment regimen was shortened to 6 months and streptomycin was replaced with ethambutol in the standard first-line regimen to avoid injections and toxicity [1, 2].

Streptomycin was reserved for re-treatment, and unfortunately has been added until recently as a single drug to failing regimens (re-treatment regimen 2), a practice originating under conditions where most treatment failures were thought to retain isoniazid and rifampin susceptibility. However, with the recognition of widespread transmission of MDR-TB in the 1980s, it became clear that many treatment failures around the world entailed loss of INH, RIF, or both key agents, and that adding a single agent could only amplify drug resistance [3].

As the approach to MDR-TB treatment became standardized in the 80s and 90s the use of other injectable drugs became routine as one of 5 categories of agents essential for a successful regimen: 1) any first-line drugs with *in vitro* susceptibility, 2) a fluoroquinolone, 3) an injectable (kanamycin, amikacin, or capreomycin), 4) one or more third-line agents, limited by effectiveness and toxicity (PAS, cycloserine, ethionamide), and 5) tertiary agents of little known efficacy, such as amoxicillin/clavulanic acid. Newer oral agents have emerged in recent years, such as moxifloxacin, linezolid, bedaquiline, and delamanid, but the same injectable agents continue to be used primarily during the early months of treatment despite toxicity, severe pain with chronic use, and difficulties with administration [4]. Exploration of alternative treatment regimens has been rather limited but is garnering greater attention given the challenges of delivering current regimens successfully at the scale needed to address the global epidemic. Among the alternative delivery systems discussed in this book, inhaled therapy is perhaps both the most promising and the most controversial. Although clinical and public health issues are often intertwined, such as toxicity impacting patient adherence, we attempt to discuss them separately.

19.3 Clinical Considerations

19.3.1 Pill Burden and Fixed-dose Combinations

Adherence to TB therapy poses many challenges including the large pill burden for many months, need for injectable therapy for drug-resistant TB, medication toxicities and drug–drug interactions. Fixed-dose combinations (FDCs) have been developed to combat

some of these issues and are recommended for the treatment of active TB in adults, particularly in resource-limited settings, provided that they are manufactured with the level of quality required to ensure adequate bioavailability of the component drugs [4]. FDCs were introduced primarily to decrease the chance of developing drug resistance due to inadvertent monotherapy in poorly adherent patients [5]. However, based on a 2013 meta-analysis, current evidence does not demonstrate improved TB treatment outcomes [6]. A 30-month follow-up from a randomized study evaluating FDCs versus separate drug regimens demonstrated that although the confidence intervals for the differences in failure/relapse rates between the two regimens were close to the pre-defined non-inferiority margin, there was a trend towards the FDC regimens performing less well than the separate drug regimens [7]. The reasons for this trend were unclear but there have been concerns about both the quality of rifampin-containing FDCs [8] and the drug bioavailability of isoniazid in FDCs [9].

19.3.2 Non-adherence and Medication Monitoring

The ability of doctors and nurses to predict non-adherence has been shown to be poor [10, 11]. Dating back to the 1960s, medication monitors (devices that record the time when medications are removed from their container) have been used to facilitate adherence outside of directly observed therapy (DOT). A study in the 1960s demonstrated that 61% of patients took 90% of their prescribed medications and 84% took greater than 70% [12]. Of note, treatment duration in this study was 18–24 months and homeless, alcoholic and psychotic patients were excluded from the study and given DOT. This study raised the question of whether medication monitors could be used to identify poorly adherent patients who are thus more likely to benefit from DOT and allow limited resources to be targeted more appropriately [11, 13]. Although medication monitors can be manipulated to create a false record, it is believed that patients with poor adherence are less likely to remember to remove the medication on time. The initial devices were cumbersome to use but improved electronic medication monitors have since been introduced, such as the Medication Event Monitoring System (MEMS), which tracks when the cap is removed from the medication bottle or records when each pill is removed from a compartment or drawer. Feedback about medication-adherence monitoring data is essential in order to enhance adherence [13] but this must happen with some frequency to be effective [14].

19.3.3 Intermittent Therapy

The need for multiple antibiotics and the duration required for TB therapy has led to considerable interest in intermittent-treatment regimens due to their practical advantages. However, a systematic review and meta-analysis demonstrated that there was insufficient evidence to support twice weekly therapy [15]. A U.S. public health service clinical trial found unacceptably high failure rates among HIV co-infected patients, leading to current U.S. guidelines specifically recommending daily therapy in that group. A Cochrane review evaluating intermittent twice- or thrice-weekly short-course regimens over daily short-course regimens determined that trial data to date are insufficient to support or refute the use of intermittent therapy for pediatric TB treatment [16]. Studies have also demonstrated an increased risk of acquired drug resistance with intermittent regimens [17].

19.3.4 Drug Toxicity

Drug toxicity is a key consideration for the clinical management of any diseases requiring multiple pharmacologic agents for extended periods of time. Side effects of anti-tubercular drugs including drug-induced hepatitis, dyspepsia, rash and arthralgia have been reported to be responsible for the termination of treatment in up to 23% of patients during the intensive therapeutic phase [18]. Medication side effects have also been often been associated with treatment interruptions and/or loss to follow-up (previously known as default) [19]. Although studies demonstrate widely varying estimates for the incidence of TB-related drug-induced liver injury (from 5–33%) [20], a study examining antimicrobials commonly used in primary-care settings demonstrated a greater-than-20-fold incidence in hepatotoxicity per 100 000 courses of isoniazid and pyrazinamide compared with other antibiotics such as quinolones [21]. Ensuring that TB programme or clinic staff are adequately trained on the recognition and management of drug-related toxicities is challenging in high-burden settings where there is limited access to lab results, such as liver-function tests, leading to delays in providers being alerted to developing hepatotoxicity.

The rise of MDR- and extensively drug-resistant (XDR)-TB poses even greater risks for drug toxicities given the need for injectable agents and even more prolonged duration of therapy. Although aminoglycoside-related nephrotoxicity is generally reversible, hearing loss is more likely to be permanent. The proportion of patients recorded to have experienced hearing loss is greatly variable with a range of <10% to >50% and a systematic approach to screening for hearing loss is lacking, particularly in high-burden settings [22]. Further work is needed to understand the risk factors for developing TB drug-induced toxicity and to develop better surveillance and reporting strategies [23].

19.3.5 Drug Absorption and Therapeutic Drug Monitoring

Amongst the myriad reasons for TB treatment failure, suboptimal drug concentrations, related to individual pharmacokinetic variability rather than non-adherence, have been associated with acquired drug resistance. This is based on both *in vitro* analyses as well as the demonstration of poor patient outcomes in prospective studies [24–26]. Patients with diabetes and those with co-existing HIV infection are at particular risk for poor drug absorption and for drug–drug interactions. Although most patients with drug-sensitive TB who are adherent with therapy will respond completely to the standard 6-month anti-TB regimen [27], certain groups of patients, including those who have MDR-TB, those who are having a slow response to treatment, those at risk for poor absorption including diabetic and HIV co-infected patients as well as those with pre-existing renal or hepatic impairment, may benefit from therapeutic drug monitoring (TDM). Although further evidence is needed to understand the clinical relevance and optimal concentrations for TB drugs, studies have demonstrated that TB drug levels are frequently below clinically acceptable levels in patients with active TB, particularly if other co-morbidities are present [28]. One unique challenge related to TB is the use of multiple-drug regimens rather than single drugs, and the testing of drug concentrations and synergies for a combination of drugs may eventually be a possibility. TDM is not part of routine practice for TB and is limited by cost and resources, as only experienced laboratories in a few settings worldwide perform this currently. Newer techniques such as dried-blood spot analysis (DBS) could allow specimens to be sent to reference laboratories without requiring special mailing cartons or

preservation of the cold chain [29]. TDM could become a valuable tool to assist clinicians in deciding whether inadequate dosing or drug–drug interactions could be causing treatment failure [30].

19.4 Public Health Considerations

When individuals are diagnosed and cured of TB, the interests of that individual and of the general public are served. However, although individuals can be cured of TB by private practitioners using their preferred treatment regimens (in the USA, at least), the public health mandate for controlling an airborne disease at the population level favours a programmatic approach. This public health approach includes standardized regimens backed by clinical trial evidence of safety and efficacy, and case management within an organized programme (often run by public health nurses) that includes the socioeconomic assessment of patients, the removal of financial and logistical barriers, treatment supervision, outreach, contact evaluation, full provider accountability, and analysis and reporting of outcomes of every case.

19.4.1 DOTS

Internationally, this approach is captured by the Directly Observed Treatment, Short course (DOTS) strategy. The five elements of DOTS are political commitment for the programme, diagnosis by quality-assured bacteriology (which is increasingly by sputum rapid molecular testing), direct supervision of a standard treatment regimen, a secure drug supply, and regular analysis of outcomes [31]. DOTS-Plus is an expansion of the DOTS programme that includes the treatment of MDR-TB, which requires access to drug-susceptibility testing and second-line drug therapy [32]. Increasingly, both drug-susceptible and -resistant TB are managed in the community by public health nurses and trained community health workers (CHWs) [33, 34]. New drug delivery systems must thus be simple enough for self-administration or community worker supervision.

Although DOTS is considered to be a core component of TB programmes, it has significant resource and cost implications that drug developers and public health policymakers must contemplate. A Cochrane Systematic Review in 2006 evaluated 10 trials with 3985 participants that compared a health worker, family member, or community volunteer routinely observing people taking anti-tubercular drugs to routine self-administration of treatment and found no difference in the number of people who were cured or completed treatment [35]. A follow-up Cochrane Review in 2015 evaluated 11 trials and stratified by DOT versus self-administered, DOT at home versus DOT at a health facility, DOT by a family member versus DOT by a CHW [36]. Again this demonstrated that overall cure and treatment-completion rates were low for both the DOT and self-treatment groups, and the authors concluded that DOT did not prove to be a solution to poor adherence to treatment. They also concluded that there was probably no difference in cure or treatment completion depending on whether DOT was administered at home or at a clinic or whether DOT was administered by a family member versus a CHW. Not usually distinguished in such studies, however, is the quality of DOT and CHW supervision, which can vary greatly between programmes, thereby reducing its apparent impact. However, the needs of vulnerable

populations such as prisoners must be considered carefully as former practices such as the self-administration of TB treatment (discontinued in 2007) was found to be associated with patients trafficking their weekend TB medications, undoubtedly driving higher rates of treatment failure and drug resistance [37]. Proponents of DOTS, however, highlight that DOT is only one part of the comprehensive case management that each TB patient requires, and stress the importance of rigorous monitoring and evaluation of patients on therapy, which is particularly pertinent for those developing new drugs and delivery systems.

19.4.2 Community-based Therapy

The first papers on successful community-based treatment for MDR-TB drew evidence from a community-based programme in Peru in the late 1990s, which demonstrated as high a percentage of probable cures as reported in any prior hospital setting, with 83% of 66 patients who were probably cured at the completion of treatment [38]. A systematic review evaluating 4 papers from Peru, the Philippines, Estonia and Tomsk Oblast in the Russian Federation found that treatment for MDR-TB could be cost effective in low- and middle-income countries, based on the cost per disability-adjusted-life-year (DALY) averted being lower than the Gross Domestic Product (GDP) per capita in all 14 of the WHO sub-regions considered [39]. Although they found that data on outpatient versus inpatient models of care was limited, their recommendation was that MDR-TB should be treated primarily in an ambulatory care setting, in the absence of good evidence for hospitalization being required to achieve high rates of adherence. Many studies have confirmed the feasibility of implementing community-based therapy for MDR- and XDR-TB in resource-limited settings [40, 41]. A systematic review of 10 studies that reported treatment outcomes from community-based MDR-TB and XDR-TB treatment programmes concluded that there were no significant differences compared with overall treatment outcomes, although there was considerable heterogeneity in terms of the type of DOT delivery site and level of community support [42]. This has led to increasing calls to promote and scale up decentralized TB care, which have implications for drug developers.

19.4.3 Incentives and Enablers to Promote Adherence

The need to provide economic support and social protection to TB patients is a concept worth emphasizing [43]. One of the new targets in WHO's End TB strategy is that no TB-affected household should experience catastrophic costs (total costs ≥20% of household annual income). This will require protection against direct and indirect health care costs as well as income losses [44]. Even when TB treatment is free, the incurring of higher relative hidden costs has been shown to adversely affect TB outcomes [45]. A review of the impact of cash transfer and microfinance schemes in low- and middle-income countries found that they could have a positive impact on TB risk factors by improving indicators of economic well-being and household food security [46].

The explosion in the use of mobile health (mHealth) technology has the potential to have a major impact on global health, particularly for infections that are treated over a prolonged period of time such as TB [47, 48]. Several medical alert systems to improve outpatient adherence using the Short Message Service (SMS) and voice features of mobile phones have been designed [49–51], and using phone call reminders to take medication has been shown to improve TB case outcomes in comparison with standard DOTS [52]. TB patients

have reported that mobile phone reminders are acceptable and helpful but barriers such as phone ownership, technical and connectivity issues and low literacy remain [53]. Despite the appeal and promise of mHealth interventions, evidence for their effectiveness is incomplete, thus WHO's Global TB Programme has developed a framework for aligning digital health interventions to its End TB strategy [54]. Incorporating mHealth and information technology solutions into novel drug delivery systems could optimize their impact.

19.5 Inhaled Drugs and Other Alternative Delivery Systems

19.5.1 Possible Advantages

Given the systemic toxicities of injectable therapy, the desirability of high lung concentrations, and recent advances in inhaled formulations and delivery systems, there is increasing interest in delivering TB drugs directly to the lungs by inhalation. Although there has been greater interest in the concept of inhaled therapies, there have been few clinical studies to demonstrate efficacy. Drugs which are poorly soluble or poorly absorbed may be especially good candidates for inhaled therapy, especially as dry-powder formulations where access to specific lung compartments can be targeted by particle size and other strategies, such as macrophage targeting. For example, as detailed below, capreomycin, normally an injectable drug, has been delivered systemically through the lungs using an absorbable nanoparticle dry-powder inhaled formulation.

The goal of inhaled-drug delivery is to enable high concentrations of pharmacologic agents in the lungs to treat pulmonary disease. As in the case of capreomycin, an important additional goal may be adequate systemic concentrations through alveolar-capillary absorption to treat extrapulmonary disease. Alternatively, to avoid systemic toxicity, possibly additive with other systemic treatment, some inhaled formulations could target lung tissue only, or primarily the airways. We detail below a recent phase 2 study of inhaled colistin (also known as Polymyxin E) intended for local deposition in the airway where aerosols are generated. In this study our purpose was to rapidly reduce the transmission of *Mycobacterium tuberculosis* (Mtb) using a unique agent not previously used for treatment of TB because of systemic toxicity at therapeutic levels. Colistin is an example of an inhaled antibiotic long used in cystic fibrosis patients to control colonization with the gram-negative bacterium *Pseudomonas aeruginosa*. Other inhaled antimicrobial agents are used for bronchiectasis, as well as for viral and fungal prophylaxis in lung transplant recipients.

Compared with nebulized drugs, inhaled dry-powder formulations of antibiotics allow more efficient delivery, and in resource-limited settings where TB is common, they negate the requirements for electricity, storage cold chains, and materials needed for reconstitution to administer as nebulized formulations.

Among clinical advantages, the large surface of the lung mucosa may enable even injectable drugs that are properly formulated to be systemically absorbed into the bloodstream without undergoing hepatic first-pass metabolism. First- or second-line TB drugs might be directed to new targets, such as alveolar macrophages that harbour TB bacilli. Inhaled drugs have been shown to achieve and maintain high drug concentrations in lung tissues, possibly utilizing companion agents that may modify host–pathogen interactions [55].

19.5.2 Concerns and Limitations

Among the first concern raised when local, inhaled therapy is proposed is the *impact of underlying abnormal lung architecture and function* on successful delivery to areas of tuberculosis disease. The underlying abnormalities may be TB-related, such as lung cavities, fibrosis, or airway destruction, or may be due to unrelated conditions such as chronic obstructive lung disease. Although inhaled dry-powder capreomycin reached systemic levels in normal volunteers, as discussed below (with likely much higher lung-tissue levels than by injection based on parallel animal studies [55]), there is no assurance that the same will be found in patients with grossly abnormal lungs. Likewise, patients with limited lung function, and children, may have difficulty producing an effective inhalation, and could experience reduced lung function due to drug- or powder-induced bronchospasm.

19.5.3 Acceptance of Novel Therapies

A fundamental question regarding inhaled TB therapy is *acceptance among providers and patients*. The Global Alliance for Drug Development conducted a survey of providers and public health officials in 5 high-burden countries concerning what they would like to see in a new first-line regimen (i.e., this was not about replacing injectable drugs) [56]. Concerning a possible inhaled first-line regimen, respondents in India, China, Kenya, and South Africa, but not Brazil, raised concerns about drug wastage and additional provider training. However, for providers treating patients with little muscle mass, especially children, facing repeated painful deep injections, it would be hard to imagine either providers or patients not preferring inhaled over injectable drugs if they worked as well or better. The tolerance and delivery efficiency of one otherwise injectable drug, capreomycin, is reviewed below. We will also mention the drug tolerance of inhaled, dry-powder colistin from the previously mentioned as-yet-unpublished clinical trial.

19.6 Clinical Trials of Inhaled Injectable Drugs

Aminoglycosides and the polypeptide, capreomycin, remain a crucial part of the MDR-TB regimen due to their *in vitro* activity against actively replicating Mtb as well as their ability to inhibit the development of drug resistance when used in combination with other drugs. Although structurally different, the polypeptide drug, capreomycin, is commonly grouped with the aminoglycosides since it has a similar spectrum of antibacterial activity and toxicity. However, the parenteral administration of aminoglycosides is associated with low lung concentrations of the drug and high serum peak levels are required to maintain microbiologically active concentrations at the most common sites of Mtb infection, which include the alveoli, pulmonary interstitium, and within macrophages [57]. Achieving adequate lung compartment-specific peak serum concentration C_{max} and systemic C_{max} (with the latter being important for extrapulmonary infection) while limiting or avoiding systemic toxicity (renal and neurologic) remains a major challenge. This has led to increasing interest in delivering medications by aerosol administration, both as a treatment and as an infection-control strategy [58].

A small non-randomized trial of 5 patients with MDR-TB demonstrated that inhaled kanamycin was safe and well tolerated, with all patients achieving sputum culture

conversion in less than 60 days [59]. Another trial of inhaled kanamycin or gentamycin alongside systemic therapy in 19 TB patients who were persistently smear- and culture-positive (despite 2 months of optimal systemic therapy) demonstrated an overall smear conversion rate of 68% after a mean duration of 58 days of aerosolized aminoglycoside therapy: 7 of 12 drug-resistant patients and 6 of 7 drug-sensitive patients. The authors noted that the therapeutic impact of inhaled aminoglycosides remained unclear since cures associated by smear conversion were not always confirmed by culture but they hypothesized that residual aminoglycoside in sputum expectorated from large cavities could prevent transmission by inhibiting intracavitary mycobacterial growth [60]. A study in Japan administered aerosolized therapy with inhaled streptomycin plus steroids and naphazoline to 20 patients with erosive or ulcerative endobronchial TB and showed a trend towards a shorter period of time to healing of the ulcerous lesions compared with historical controls treated with standard oral anti-tuberculosis chemotherapy [61]. A follow-up prospective study by the same authors administered the same combination of inhaled streptomycin plus steroids and naphazoline to 30 patients with endobronchial TB and noted that three demonstrated improvement and 27 demonstrated no progression of disease, whereas of the 27 patients who received conventional therapy only one improved, 13 demonstrated no change and 13 demonstrated worsening endoscopic findings. This suggested that combination aerosol therapy could play a role in the treatment of endobronchial TB [62].

Capreomycin has also been shown to have activity against non-replicating forms of Mtb [63]. It is typically administrated in intravenous or intramuscular form, at a usual dose of 15–20 mg/kg of body weight/day for at least a 6-month period for treatment of MDR- or XDR-TB, although treatment for up to 2 years may be needed if it is tolerated. A study evaluating adverse effects of MDR-TB therapy in Tomsk, Russia, demonstrated that 38 of 244 patients (15.6%) developed ototoxicity (defined as hearing loss confirmed by physical examination or audiometry). 13 patients (34.2% of the 38 with ototoxicity) required permanent interruption of the drug to which the hearing loss was attributed (12 due to kanamycin and 1 due to capreomycin) [64]. A study of 50 patients initiated on injectable aminoglycosides for MDR-TB therapy in the UK demonstrated that 14 (28%) developed ototoxicity with 9 (18%) left with long-term hearing loss, none of whom were on capreomycin, but the numbers were too small to allow conclusions to be drawn [65]. Although there are even fewer data regarding the effect of inhaled aminoglycosides on hearing loss, phase 3 trial data evaluating inhaled tobramycin did not demonstrate high-frequency hearing loss in the treatment arm compared with the placebo arm [66]. A study evaluating cystic fibrosis patients did not demonstrate that hearing loss was more frequent in those patients who had received aminoglycoside therapy, although this included both intravenous and inhaled formulations [67]. The likelihood of significant nephrotoxicity due to inhaled aminoglycosides is also thought to be low and a review of inhaled tobramycin in cystic fibrosis patients noted that nephrotoxicity and hearing loss had not occurred in clinical trials although transient mild or moderate tinnitus occurred more often in the patients who received inhaled tobramycin compared with those who received placebo [68].

Pre-clinical testing of a dry-powder microparticle formulation of capreomycin demonstrated that it exhibited good aerosolization properties, physical-chemical stability, efficient lung deposition and absorption. Experimental studies in rats and dogs showed some mild, reversible dose-dependent histopathological respiratory tract irritation with pharmacokinetic studies in guinea pigs demonstrating efficient drug absorption from the lung into

blood and a linear terminal phase of the plasma concentration–time curve. Studies of dry-powder capreomycin in guinea pigs additionally demonstrated that the systemic concentrations achieved after a 14.5 mg/kg inhaled dose were comparable to systemic concentrations seen 2 hours after a 14.5 mg/kg dose was given by intravenous (i.v.) or intramuscular (i.m.) routes. Additionally with inhaled drug delivery, the high initial spike in systemic concentrations seen with i.v. or i.m. administration was not produced. Daily administration of the inhaled dose at a dose of 14.5 mg/kg over a 4-week period resulted in significant reductions in bacterial burden and lung pathology in Mtb-infected guinea pigs, which represented a greater pharmacodynamics effect on lung bacterial burden than the i.m. dose of 20 mg/kg achieved. The longer systemic half-life observed after a single inhaled dose also demonstrates superiority over parenterally administered capreomycin in terms of achieving therapeutic pulmonary concentrations [55].

19.6.1 Capreomycin Phase 1 Clinical Study

A phase 1 clinical study of inhaled capreomycin was conducted among 20 healthy adult volunteers in Boston, USA [69]. This study used capreomycin in inhaled doses ranging from doses 22-fold lower than the no-adverse-effect level (NOAEL) in dogs (for the 25-mg group) to 1.8-fold lower than the NOAEL in dogs (for the 300-mg group). Subjects were assigned to one of four escalating dosage groups (25, 75, 150 and 300 mg capreomycin with 5 subjects per group). The dose was self-administered with a handheld inhaler into which capsules containing 25 mg of capreomycin and 5 mg of the excipient L-leucine, at an 80:20 ratio, were inserted sequentially. Subjects underwent blood sampling for pharmacokinetic analysis at 13 time points: pre-dose; 10, 20, 30, and 45 min post-dose; as well as 1, 2, 3, 4, 6, 8, 12, and 24 h post-dose. Drug concentrations were assayed by using a highly sensitive high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) methodology. The lower limit of quantitation of capreomycin in human plasma was 100 ng/ml, with an accuracy of 88.2%, and the linear range of quantitation was 100 to 5000 ng/ml.

Capreomycin was detectable in serum samples within 20 minutes of inhalation in 17/20 (85%) of study patients and within 1–3 hours in the remaining 3 subjects who were in the 25 mg lowest-dose group. Both the maximal plasma concentration (C_{max}) and the area under the curve (AUC) from 0 hours to infinity ($AUC_{0-\infty}$) were dose proportional. The mean C_{max} values for each successive dose group were 169, 569, 972 and 2315 ng/ml. The mean area under the concentration–time curves from 0 hours to the last measurable concentration (AUC_{0-t}) ranged from 969 h · ng/ml in the 25 mg group to 19 959 h · ng/ml in the 300 mg group. At the highest dose of 300 mg, after a single dose the mean plasma concentration exceeded the minimum inhibitory concentration (MIC) of 2 μg/ml (or 2000 ng/ml) for *M. tuberculosis* based on values reported in other studies.

Inhaled capreomycin was well tolerated. There were no changes in lung function, audiometry or other clinical or laboratory parameters. There were no serious or severe adverse effects and the most common adverse event was mild to moderate transient cough, which occurred in five subjects. However, this was a single-dose study. Repeated dose studies have yet to be done.

The 300 mg dose of capreomycin used in this study was below the estimate of the NOAEL determined through pre-clinical testing in a canine model, so higher doses may

safely be used to produce even higher C_{max} and AUC. However, a 300 mg dose here required sequential inhalation of twelve 25 mg capsules, although this could be reduced by half with fully loaded capsules. To achieve the typical 15 mg/kg capreomycin dose typically used in adults may be logistically challenging in terms of the powder load and dose frequency. Even 300 mg is a large dose for inhaled drugs. As mentioned, divided-dose therapy is usually avoided in the management of drug-resistant TB due to full supervision of medication ingestion being the standard of care. Nonetheless, inhaled capreomycin could be a valuable addition to MDR-TB management, particularly in the setting of other drug interactions including cumulative nephro- and oto-toxicity and for pediatric MDR-TB where the hardships of injectable drugs are incrementally more difficult. Study participants in this phase 1 study were predominantly healthy males and so the pharmacokinetics of inhaled drugs in patients with lung disease may be quite different. Further studies to evaluate the pharmacokinetics and pharmacodynamics of higher doses of inhaled capreomycin in healthy and MDR-TB patients are needed. However, parallel studies in animals indicate much higher lung-tissue concentrations by inhaled compared with injectable administration, suggesting that, at least in the lungs, lower doses may suffice with less systemic toxicity, as long as systemic infection is treated by other agents [70].

19.6.2 Inhaled Therapy to Reduce Transmission, especially of Highly Drug-resistant Strains – a Trial of Inhaled Colistin (or Polymxyin E)

The treatment of isoniazid- and rifampin-resistant TB (MDR) results in Mtb exposure to a wide range of antibiotics and the inevitable selection of additional drug resistance beyond MDR-TB [5]. When resistance to the next two most important classes of drugs, fluoroquinolones and the injectables (beyond streptomycin), has occurred, treatment success falls markedly for what is then called XDR-TB. The remaining drugs have had some success in some reports, but, overall, success rates are low, especially when TB is co-incident with HIV infection [71]. The recent availability of two new antibiotics, bedaquiline and delamanid, will save many lives, but in time resistance to these drugs too will emerge with their use. XDR (and some MDR) patients who remain sputum smear- or culture-positive after years of treatment present medical, ethical, and logistical problems for their families and the community. Lifelong hospitalization for infection control is ethically problematic, and only practical in pre-terminal patients. Living with families and contact with the community risks transmission of a potentially deadly infection. Masks on patients have been shown to be only about 50% effective in preventing transmission [72]. Separate living quarters have been arranged as a partial solution.

Researchers in the US and South Africa envisioned the use of a novel, non-toxic inhaled agent to prevent transmission by locally inactivating organisms in the airways before they are aerosolized. Colistin (Polymyxin E) was chosen because its safety was well established as an inhaled antibiotic to control bacterial infection in the lungs of cystic fibrosis (CF) patients – often with markedly impaired lung function. Originally nebulized, colistin is now commercially available as a dry-powder delivery system. Colistin had decades ago been tested *in vitro* as an antimycobacterial and was found to have broad, mild to modest activity, but, because of renal toxicity worse than that found with existing injectable agents, it was never used parenterally, and its potential utility was forgotten. However, pharmacodynamic studies of inhaled colistin in CF patients showed minimal systemic absorption.

Since colistin has never been used for TB therapy, resistance by even highly drug-resistant strains is unlikely, and the high local concentrations in the lungs, especially using dry-powder inhalation, would likely far exceed the relatively high MIC for Mtb. Finally, colistin has a unique detergent-like mode of action, disrupting cell walls, with demonstrable synergy with some other drugs against both pseudomonas and mycobacteria [73]. Other *in vitro* studies have demonstrated a greater killing effect when colistin was used in conjunction with isoniazid, with ultrastructure analysis suggesting that this is due to disruption of the outer polysaccharide layer of Mtb by colistin, which increases isoniazid uptake [74].

With the exclusive goal of reducing transmission, rather than individualized patient therapy, a phase 2 clinical trial of dry-powder inhaled colistin was undertaken in an MDR/XDR TB referral centre in Mpumalanga, South Africa. Study-design problems are now appreciated, and the study has not yet been published. The following summary data have been presented at scientific meetings [75].

Over a period of 4 months, 6 MDR or XDR patients per month (24 patients in total) were recruited to occupy a special experimental ward designed to study human-to-guinea pig Mtb transmission. Guinea pigs are highly susceptible to human tuberculosis in the low doses found in the exhaust air of this special facility, and the human-to-guinea pig model has been used effectively to test a variety of control interventions [76]. In addition to their standard systemic TB treatment, these patients consented to inhale the same dose of dry-powder colistin that had been safely administered every 8 hours for CF patients in clinical trials. The flawed study design was as follows. Over the course of 4 weeks, the first cohort of 6 patients received one week of their standard oral and injected MDR-TB treatment (no inhaled colistin), and exhaust air exposed only "control" chamber guinea pigs. The 2nd week the same patients received inhaled colistin in addition to systemic therapy, and exhaust air exposed only the guinea pigs in the "intervention" chamber. The 3rd and 4th weeks were exact repeats of weeks 1 and 2 with the same patients – a critical difference being that by control week 3, the patients had already been exposed to inhaled colistin since week 2, and there were likely (unwanted) carryover effects of the inhaled drug. The rationale for this alternate week colistin treatment/control protocol was to assure that temporal changes in patient infectiousness due to systemic treatment would not be interpreted as inhaled-drug effect. It assumed no carryover effects. Moreover, the second 4-week cohort of patients started with inhaled colistin, potentially compromising both "control" periods that followed due to possible carryover inhaled-drug effect. Although inconclusive, the results of this study are nonetheless interesting. At month 1 there were 18 infections in the guinea pig chamber exposed to patients who had at least week 1 of systemic therapy only – no inhaled colistin. There were only 8 infections (56% fewer) among guinea pigs exposed to patients who had had inhaled colistin weeks 2 and 4 – with probable carryover effects of inhaled drug during week 3. For patient cohort 3, when there was again a 1-week true control period, with no inhaled drug, 3 infections occurred whereas only 1 infection occurred in the "intervention" chamber. As noted, for patient cohorts 2 and 4, there were no true control weeks if there was any carryover effect from week 1 colistin use. Overall, the study was inconclusive for proving a beneficial effect, but the expected result of an unanticipated carryover effect would be to reduce transmission in most control weeks, reducing the difference compared with intervention weeks. We hope to repeat this important study, anticipating a prolonged drug effect after inhalation and employing a different study design.

19.7 Other Novel Delivery Strategies

There is increasing interest in the possibility of anti-TB drugs being incorporated into liposomes, microparticles or nanoparticles, which can be delivered in dry-powder form to enable instantaneous, targeted and/or controlled release directly into the lungs. Nanoparticles have been previously described in this book and evaluation of these investigational therapies in animals has demonstrated decreases in the numbers of viable bacteria as well as reduced tissue damage. Potential clinical advantages of these new therapies aside from improved efficacy due to improved pulmonary drug distribution include reduced doses and frequency of administration, which could improve patient adherence and decrease systemic toxicity. Particularly for patients with MDR-TB who currently require months of intramuscular aminoglycoside injections, the non-invasive route of administration for these new technologies is a major advantage [77, 78].

Other novel strategies include subcutaneous drug delivery systems. A subdermal multidrug reservoir is designed to contain TB drugs for the maintenance phase of therapy when lower but sustained drug levels are needed. One such technology is based on encapsulating isoniazid and/or rifampin in a biodegradable polymeric matrix that allows slow controlled drug release [79]. This could potentially have a major impact on improving treatment adherence and decreasing acquired drug resistance. Potential barriers include the cost of the technology and the need to train healthcare workers to be able to administer such therapies, as well as convincing patients and health care workers of the benefits of a subdermal drug delivery system compared with the known entity that oral medications represent. Safety is a major concern. A patient who is sick with isoniazid-induced hepatitis is likely not to take more drug at some point, but depot systems will continue to deliver the drug, potentially leading to irreversible toxicity.

19.8 Pediatric Delivery Systems

Children are often overlooked in the global response to the TB and MDR-TB pandemics, despite contributing significantly to the caseload [80]. There is a lack of accurate diagnostic tools as well as pharmacokinetic data to guide dosing recommendations for children. Additional challenges include the considerable changes that occur in the absorption, distribution and excretion of medications that occur between birth and adolescence, as well as the dramatic changes in body surface area and total body water that occur between infancy and adulthood. Pharmacokinetic data demonstrate that children receiving certain TB drugs, including isoniazid, are exposed to lower serum concentrations of these drugs than are adults [81]. HIV-positive children may also be exposed to lower concentrations than age-matched HIV-negative children, often related to interactions with concurrent anti-retroviral therapy [82]. Infants have immature enzyme systems which can expose them to drug toxicity. Given that the diagnosis of childhood TB is complicated by the lack of objective data due to the lower proportion of microbiologically confirmed disease and specific radiographic signs such as cavitation seen in children compared with adults, assessing children's response to pharmacologic therapy can be challenging [83].

There is a distinct lack of child-friendly drug delivery systems for TB therapy, particularly for second-line drugs required for MDR-TB therapy. Although syrup formulations

exist for some second-line medications such as linezolid and levofloxacin, this can be cumbersome as large volumes may be required daily. Pain from injectable agents is a considerable barrier for pediatric TB therapy. Manufacturers do not recommend breaking and crushing tablets, opening capsules or compounding drugs due to unpredictable pharmacokinetic effects. Many second-line TB drugs are not licensed for use in children and must be used off-label. Suspensions often require refrigeration and have shorter half-lives and so are less preferable to dispersible tablets or granules. In addition to increased stability compared with liquid formulations, granular formulations may also mask the unpleasant taste of medications. Although fixed-dose combination dispersible tablets have been developed for other diseases such as HIV, this may take decades to adapt for pediatric MDR-TB therapy given the lack of certainty regarding the doses and number of drugs needed in this setting.

A granular PAS dosing spoon has been developed as an innovative alternative pediatric drug delivery system. PAS was one of the first TB medications but its use was limited by large pill size and pill burden, prompting development of a gastro-resistant granular form known as Paser^{R}, which comes in 4 g sachets. Pediatric dosing is made challenging by the need to weigh out granules in order to provide the necessary 150–300 mg/kg daily doses. Lucane, a French pharmaceutical company, developed a pediatric dosing spoon calibrated for Paser^{R}. This allows providers to dispense appropriate doses of Paser^{R} to children based on cut-off marks for the different PAS doses based on weight-band doses. Challenges that were encountered in developing this novel delivery system include developing an adequate level of calibration and accuracy, and dispensing doses that are between the calibrated doses. However, since FDC solutions will take some time to develop, and there is an urgent need for child-friendly TB drug delivery systems, a granular pediatric dosing spoon is a strategy that could also be applied to cycloserine or ethionamide and could be a valuable addition to our current practice [84].

19.9 Conclusion

There is a pressing need for improved TB therapeutic regimens that have less systemic toxicities, particularly for MDR-TB. Novel inhaled drug delivery systems hold much promise if they can additionally address the clinical and public health challenges raised in this discussion.

References

[1] Daniel, T.M. (2006) The history of tuberculosis. *Respiratory Medicine*, **100**(11), 1862–1870. PubMed PMID: 16949809.
[2] Iseman, M.D. (2002) Tuberculosis therapy: past, present and future. *European Respiratory Journal*, **36** (Supplement), 87s–94s. PubMed PMID: 12168751.
[3] Nachega, J.B. and Chaisson, R.E. (2003) Tuberculosis drug resistance: a global threat. *Clinical Infectious Diseases*, **36**(Suppl. 1), S24–S30. PubMed PMID: 12516027.
[4] WHO 2010 Guidelines for treatment of tuberculosis. 4th edn.
[5] Moulding, T., Dutt, A.K. and Reichman, L.B. (1995) Fixed-dose combinations of antituberculous medications to prevent drug resistance. *Annals of Internal Medicine*, **122**(12), 951–954. PubMed PMID: 7755232.

[6] Albanna, A.S., Smith, B.M., Cowan, D. and Menzies, D. (2013) Fixed-dose combination antituberculosis therapy: a systematic review and meta-analysis. *European Respiratory Journal*, **42**(3), 721–732. PubMed PMID: 23314904.

[7] Nunn, A.J., Cook, S.V., Burgos, M. *et al.* (2014) Results at 30 months of a randomised trial of FDCs and separate drugs for the treatment of tuberculosis. *International Journal of Tuberculosis and Lung Disease*, **18**(10), 1252–1254. PubMed PMID: 25216842.

[8] Ellard, G.A. (1999) The evaluation of rifampicin bioavailabilities of fixed-dose combinations of anti-tuberculosis drugs: procedures for ensuring laboratory proficiency. *International Journal of Tuberculosis and Lung Disease*, **3**(11 Suppl. 3), S322–S324; discussion S51–S52. PubMed PMID: 10593711.

[9] Xu, J., Jin, H., Zhu, H. *et al*. (2013) Oral bioavailability of rifampicin, isoniazid, ethambutol, and pyrazinamide in a 4-drug fixed-dose combination compared with the separate formulations in healthy Chinese male volunteers. *Clinical Therapeutics*, **35**(2), 161–168. PubMed PMID: 23410999.

[10] Sackett, D.L. Haynes, R.B., Guyatt, G.H. and Tugwell, P. (1991) *Clinical Epidemiology: A basic science for clinical medicine*, 2nd edn, Boston, Little, Brown.

[11] Moulding, T.S. (1979) The unrealized potential of the medication monitor. *Clinical Pharmacology and Therapeutics*, **25**(2), 131–136. PubMed PMID: 759065.

[12] Moulding, T., Onstad, G.D. and Sbarbaro, J.A. (1970) Supervision of outpatient drug therapy with the medication monitor. *Annals of Internal Medicine*, **73**(4), 559–564. PubMed PMID: 4319078.

[13] Moulding, T.S. and Caymittes, M. (2002) Managing medication compliance of tuberculosis patients in Haiti with medication monitors. *International Journal of Tuberculosis and Lung Disease*, **6**(4), 313–319. PubMed PMID: 11936740.

[14] Fallab-Stubi, C.L., Zellweger, J.P., Sauty, A. *et al.* (1998) Electronic monitoring of adherence to treatment in the preventive chemotherapy of tuberculosis. *International Journal of Tuberculosis and Lung Disease*, **2**(7), 525–530. PubMed PMID: 9661817.

[15] Menzies, D., Benedetti, A., Paydar, A. *et al*. (2009) Effect of duration and intermittency of rifampin on tuberculosis treatment outcomes: a systematic review and meta-analysis. *PLoS Medicine*, **6**(9), e1000146. PubMed PMID: 19753109. Pubmed Central PMCID: 2736385.

[16] Bose, A., Kalita, S., Rose, W. and Tharyan, P. (2014) Intermittent versus daily therapy for treating tuberculosis in children. *Cochrane Database of Systematic Reviews* **1** (Art. No.: CD007953). PubMed PMID: 24470141.

[17] Narendran, G., Menon, P.A., Venkatesan, P. *et al.* (2014) Acquired rifampicin resistance in thrice-weekly antituberculosis therapy: impact of HIV and antiretroviral therapy. *Clinical Infectious Diseases*, **59**(12), 1798–1804. PubMed PMID: 25156114.

[18] Schaberg, T., Rebhan, K. and Lode, H. (1996) Risk factors for side-effects of isoniazid, rifampin and pyrazinamide in patients hospitalized for pulmonary tuberculosis. *European Respiratory Journal*, **9**(10), 2026–2030. PubMed PMID: 8902462.

[19] Tekle, B., Mariam, D.H. and Ali, A. (2002) Defaulting from DOTS and its determinants in three districts of Arsi Zone in Ethiopia. *International Journal of Tuberculosis and Lung Disease*, **6**(7), 573–579. PubMed PMID: 12102295.

[20] Saukkonen, J.J., Cohn, D.L., Jasmer, R.M. *et al*. (2006) An official ATS statement: hepatotoxicity of antituberculosis therapy. *American Journal of Respiratory and Critical Care Medicine*, **174**(8), 935–952. PubMed PMID: 17021358.

[21] Andrade, R.J. and Tulkens, P.M. (2011) Hepatic safety of antibiotics used in primary care. *Journal of Antimicrobial Chemotherapy*, **66**(7), 1431–1446. PubMed PMID: 21586591. Pubmed Central PMCID: 3112029.

[22] Seddon, J.A., Godfrey-Faussett, P., Jacobs, K. *et al*. (2012) Hearing loss in patients on treatment for drug-resistant tuberculosis. *European Respiratory Journal*, **40**(5), 1277–1286. PubMed PMID: 22700838.

[23] Frydenberg, A.R. and Graham, S.M. (2009) Toxicity of first-line drugs for treatment of tuberculosis in children: review. *Tropical Medicine & International Health*, **14**(11), 1329–1337. PubMed PMID: 19735381.

[24] Srivastava, S., Pasipanodya, J.G., Meek, C. *et al*. (2011) Multidrug-resistant tuberculosis not due to noncompliance but to between-patient pharmacokinetic variability. *Journal of Infectious Diseases*, **204**(12), 1951–1959. PubMed PMID: 22021624. Pubmed Central PMCID: 3209814.

[25] Pasipanodya, J.G., McIlleron, H., Burger, A. *et al*. (2013) Serum drug concentrations predictive of pulmonary tuberculosis outcomes. *Journal of Infectious Diseases*, **208**(9), 1464–1473. PubMed PMID: 23901086. Pubmed Central PMCID: 3789573.

[26] Chideya, S., Winston, C.A., Peloquin, C.A. *et al.* (2009) Isoniazid, rifampin, ethambutol, and pyrazinamide pharmacokinetics and treatment outcomes among a predominantly HIV-infected cohort of adults with tuberculosis from Botswana. *Clinical Infectious Diseases*, **48**(12), 1685–1694. PubMed PMID: 19432554. Pubmed Central PMCID: 3762461.

[27] Blumberg, H.M., Burman, W.J., Chaisson, R.E. *et al*. (2003) American Thoracic Society/ Centers for Disease Control and Prevention/Infectious Diseases Society of America: treatment of tuberculosis. *American Journal of Respiratory and Critical Care Medicine*, **167**(4), 603–662. PubMed PMID: 12588714.

[28] Babalik, A., Mannix, S., Francis, D. and Menzies, D. (2011) Therapeutic drug monitoring in the treatment of active tuberculosis. *Canadian Respiratory Journal*, **18**(4), 225–229. PubMed PMID: 22059181. Pubmed Central PMCID: 3205104.

[29] Edelbroek, P.M., van der Heijden, J. and Stolk, L.M. (2009) Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. *Therapeutic Drug Monitoring*, **31**(3), 327–336. PubMed PMID: 19349929.

[30] Alsultan, A. and Peloquin, C.A. (2014) Therapeutic drug monitoring in the treatment of tuberculosis: an update. *Drugs*, **74**(8), 839–854. PubMed PMID: 24846578.

[31] Lienhardt, C., Glaziou, P., Uplekar, M. *et al.* (2012) Global tuberculosis control: lessons learnt and future prospects. *Nature Reviews Microbiology*, **10**(6), 407–416. PubMed PMID: 22580364.

[32] Farmer, P. and Kim, J.Y. (1998) Community based approaches to the control of multidrug resistant tuberculosis: introducing "DOTS-plus". *BMJ*, **317**(7159), 671–674. PubMed PMID: 9728004. Pubmed Central PMCID: 1113843.

[33] Chaulk, C.P., Moore-Rice, K., Rizzo, R. and Chaisson, R.E. (1995) Eleven years of community-based directly observed therapy for tuberculosis. *JAMA: Journal of the American Medical Association*, **274**(12), 945–951. PubMed PMID: 7674524.

[34] Wright, C.M., Westerkamp, L., Korver, S. and Dobler, C.C. (2015) Community-based directly observed therapy (DOT) versus clinic DOT for tuberculosis: a systematic review and meta-analysis of comparative effectiveness. *BMC Infectious Diseases*, **15**, 210. PubMed PMID: 25948059. Pubmed Central PMCID: 4436810.

[35] Volmink, J. and Garner, P. (2006) Directly observed therapy for treating tuberculosis. *Cochrane Database of Systematic Reviews* **2** (Art. No.: CD003343). PubMed PMID: 16625579.

[36] Karumbi, J. and Garner, P. (2015) Directly observed therapy for treating tuberculosis. *Cochrane Database of Systematic Reviews* **5** (Art. No.: CD003343). PubMed PMID: 26022367. Pubmed Central PMCID: 4460720.

[37] Awofeso, N. (2008) Anti-tuberculosis medication side-effects constitute major factor for poor adherence to tuberculosis treatment. *Bulletin of the World Health Organization*, **86**(3), B–D. PubMed PMID: 18368191. Pubmed Central PMCID: 2647396.

[38] Mitnick, C., Bayona, J., Palacios, E. *et al*. (2003) Community-based therapy for multidrug-resistant tuberculosis in Lima, Peru. *New England Journal of Medicine*, **348**(2), 119–128. PubMed PMID: 12519922.

[39] Fitzpatrick, C. and Floyd, K. (2012) A systematic review of the cost and cost effectiveness of treatment for multidrug-resistant tuberculosis. *PharmacoEconomics*, **30**(1), 63–80. PubMed PMID: 22070215.

[40] Satti, H., McLaughlin, M.M., Hedt-Gauthier, B. *et al*. (2012) Outcomes of multidrug-resistant tuberculosis treatment with early initiation of antiretroviral therapy for HIV co-infected patients in Lesotho. *PLoS One*, **7**(10), e46943. PubMed PMID: 23115633. Pubmed Central PMCID: 3480376.

[41] Heller, T., Lessells, R.J., Wallrauch, C.G. *et al*. (2010) Community-based treatment for multidrug-resistant tuberculosis in rural KwaZulu-Natal, South Africa. *International Journal of Tuberculosis and Lung Disease*, **14**(4), 420–426. PubMed PMID: 20202299.

[42] Weiss, P., Chen, W., Cook, V.J. and Johnston, J.C. (2014) Treatment outcomes from community-based drug resistant tuberculosis treatment programs: a systematic review and meta-analysis. *BMC Infectious Diseases*, **14**, 333. PubMed PMID: 24938738. Pubmed Central PMCID: 4071022.

[43] Richter, L.M., Lonnroth, K., Desmond, C. *et al*. (2014) Economic support to patients in HIV and TB grants in rounds 7 and 10 from the global fund to fight AIDS, tuberculosis and malaria. *PLoS One*, **9**(1), e86225. PubMed PMID: 24489702. Pubmed Central PMCID: 3904874.

[44] Lonnroth, K. and Weil, D.E. (2014) Mass prophylaxis of tuberculosis through social protection. *The Lancet Infectious Diseases*, **14**(11), 1032–1034. PubMed PMID: 25303846.

[45] Wingfield, T., Boccia, D., Tovar, M. *et al*. (2014) Defining catastrophic costs and comparing their importance for adverse tuberculosis outcome with multi-drug resistance: a prospective cohort study, Peru. *PLoS Medicine*, **11**(7), e1001675. PubMed PMID: 25025331. Pubmed Central PMCID: 4098993.

[46] Boccia, D., Hargreaves, J., Lonnroth, K. *et al.* (2011) Cash transfer and microfinance interventions for tuberculosis control: review of the impact evidence and policy implications. *International Journal of Tuberculosis and Lung Disease*, **15**(Suppl. 2), S37–S49. PubMed PMID: 21740658. Pubmed Central PMCID: 3160484.

[47] Denkinger, C.M., Grenier, J., Stratis, A.K. *et al*. (2013) Mobile health to improve tuberculosis care and control: a call worth making. *International Journal of Tuberculosis and Lung Disease*, **17**(6), 719–727. PubMed PMID: 23541232.

[48] Devi, B.R., Syed-Abdul, S., Kumar, A. *et al*. (2015) mHealth: An updated systematic review with a focus on HIV/AIDS and tuberculosis long term management using mobile phones. *Computer Methods and Programs in Biomedicine*, Aug 10. PubMed PMID: 26304621.

[49] Bediang, G., Stoll, B., Elia, N. *et al*. (2014) SMS reminders to improve the tuberculosis cure rate in developing countries (TB-SMS Cameroon): a protocol of a randomised control study. *Trials*, **15**, 35. PubMed PMID: 24460827. Pubmed Central PMCID: 3902069.

[50] Iribarren, S.J., Sward, K.A., Beck, S.L. *et al.* (2015) Qualitative evaluation of a text messaging intervention to support patients with active tuberculosis: implementation considerations. *JMIR mHealth and uHealth*, **3**(1), e21. PubMed PMID: 25802968. Pubmed Central PMCID: 4376194.

[51] Okuboyejo, S. and Eyesan, O. (2014) mHealth: Using Mobile Technology to Support Healthcare. *Online Journal of Public Health Informatics*, **5**(3), 233. PubMed PMID: 24678384. Pubmed Central PMCID: 3959917.

[52] Kunawararak, P., Pongpanich, S., Chantawong, S. *et al*. (2011) Tuberculosis treatment with mobile-phone medication reminders in northern Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health*, **42**(6), 1444–1451. PubMed PMID: 22299414.

[53] Mohammed, S., Siddiqi, O., Ali, O. *et al*. (2012) User engagement with and attitudes towards an interactive SMS reminder system for patients with tuberculosis. *Journal of Telemedicine and Telecare*, **18**(7), 404–408. PubMed PMID: 23034935.

[54] Falzon, D., Raviglione, M., Bel, E.H. *et al*. (2015) The role of eHealth and mHealth in tuberculosis and tobacco control: a WHO/ERS consultation. *European Respiratory Journal*, **46**(2), 307–311. PubMed PMID: 26232477.

[55] Garcia-Contreras, L., Fiegel, J., Telko, M.J. *et al.* (2007) Inhaled large porous particles of capreomycin for treatment of tuberculosis in a guinea pig model. *Antimicrobial Agents and Chemotherapy*, **51**(8), 2830–2836. PubMed PMID: 17517845. Pubmed Central PMCID: 1932523.

[56] Global TB Alliance (August 2009) Value Proposition Study. New TB Regimens: What Countries Want. The Value Proposition of Existing and New First-Line Regimens for Drug-Susceptible Tuberculosis. http://www.tballiance.org/downloads/publications/TBA_VPSreport_final_flat.pdf (accesesed 11th May 2016).

[57] Carcas, A.J., Garcia-Satue, J.L., Zapater, P. and Frias-Iniesta, J. (1999) Tobramycin penetration into epithelial lining fluid of patients with pneumonia. *Clinical Pharmacology and Therapeutics*, **65**(3), 245–250. PubMed PMID: 10096256.

[58] Pandey, J.S., Khan, S., Joseph, V. and Kumar, R. (2002) Aerosol scavenging: model application and sensitivity analysis in the Indian context. *Environmental Monitoring and Assessment*, **74**(2), 105–116. PubMed PMID: 11878638.

[59] Turner, M.T., Haskal, R., McGowan, K. *et al*. (1998) Inhaled Kanamycin in the Treatment of Multidrug-Resistant Tuberculosis: A Study of Five Patients. *Infectious Diseases in Clinical Practice*, **7**(1), 49–53.

[60] Sacks, L.V., Pendle, S., Orlovic, D. *et al.* (2001) Adjunctive salvage therapy with inhaled aminoglycosides for patients with persistent smear-positive pulmonary tuberculosis. *Clinical Infectious Diseases*, **32**(1), 44–49. PubMed PMID: 11118385.

[61] Rikimaru, T., Kinosita, M., Yano, H. *et al*. (1998) Diagnostic features and therapeutic outcome of erosive and ulcerous endobronchial tuberculosis. *International Journal of Tuberculosis and Lung Disease*, **2**(7), 558–562. PubMed PMID: 9661822.

[62] Rikimaru, T., Koga, T., Sueyasu, Y. *et al.* (2001) Treatment of ulcerative endobronchial tuberculosis and bronchial stenosis with aerosolized streptomycin and steroids. *International Journal of Tuberculosis and Lung Disease*, **5**(8), 769–774. PubMed PMID: 11495269.

[63] Heifets, L., Simon, J. and Pham, V. (2005) Capreomycin is active against non-replicating M. tuberculosis. *Annals of Clinical Microbiology and Antimicrobials*, **4**, 6. PubMed PMID: 15804353. Pubmed Central PMCID: 1083412.

[64] Shin, S.S., Pasechnikov, A.D., Gelmanova, I.Y. *et al*. (2007) Adverse reactions among patients being treated for MDR-TB in Tomsk, Russia. *International Journal of Tuberculosis and Lung Disease*, **11**(12), 1314–1320. PubMed PMID: 18034952.

[65] Sturdy, A., Goodman, A., Jose, R.J. *et al*. (2011) Multidrug-resistant tuberculosis (MDR-TB) treatment in the UK: a study of injectable use and toxicity in practice. *Journal of Antimicrobial Chemotherapy*, **66**(8), 1815–1820. PubMed PMID: 21642291.

[66] Gibson, R.L., Burns, J.L. and Ramsey, B.W. (2003) Pathophysiology and management of pulmonary infections in cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine*, **168**(8), 918–951. PubMed PMID: 14555458.

[67] Martins, L.M., Camargos, P.A., Becker, H.M. *et al*. (2010) Hearing loss in cystic fibrosis. *International Journal of Pediatric Otorhinolaryngology*, **74**(5), 469–473. PubMed PMID: 20189661.

[68] Cheer, S.M., Waugh, J. and Noble, S. (2003) Inhaled tobramycin (TOBI): a review of its use in the management of Pseudomonas aeruginosa infections in patients with cystic fibrosis. *Drugs*, **63**(22), 2501–2520. PubMed PMID: 14609360.

[69] Dharmadhikari, A.S., Kabadi, M., Gerety, B. *et al.* (2013) Phase I, single-dose, dose-escalating study of inhaled dry powder capreomycin: a new approach to therapy of drug-resistant tuberculosis. *Antimicrobial Agents and Chemotherapy*, **57**(6), 2613–2619. PubMed PMID: 23529740. Pubmed Central PMCID: 3716148.

[70] Hickey, A.J. and Garcia-Contreras, L. (2001) Immunological and toxicological implications of short-term studies in animals of pharmaceutical aerosol delivery to the lungs: relevance to humans. *Critical Reviews in Therapeutic Drug Carrier Systems*, **18**(4), 387–431. PubMed PMID: 11605897.

[71] Gunther, G. (2014) Multidrug-resistant and extensively drug-resistant tuberculosis: a review of current concepts and future challenges. *Clinical Medicine*, **14**(3), 279–285. PubMed PMID: 24889573.

[72] Dharmadhikari, A.S., Mphahlele, M., Stoltz, A. *et al*. (2012) Surgical face masks worn by patients with multidrug-resistant tuberculosis: impact on infectivity of air on a hospital ward. *American Journal of Respiratory and Critical Care Medicine*, **185**(10), 1104–1109. PubMed PMID: 22323300. Pubmed Central PMCID: 3359891.

[73] Bax, H.I., de Steenwinkel, J.E., Ten Kate, M.T. *et al.* (2015) Colistin as a potentiator of anti-TB drug activity against Mycobacterium tuberculosis. *Journal of Antimicrobial Chemotherapy*, **70**(10), 2828–2837. PubMed PMID: 26183185.

[74] van Breda, S.V., Buys, A., Apostolides, Z. *et al*. (2015) The antimicrobial effect of colistin methanesulfonate on Mycobacterium tuberculosis in vitro. *Tuberculosis (Edinburgh, Scotland)*, **95**(4), 440–446. PubMed PMID: 26038290.

[75] Nardell, E.A., Stoltz, A. and the AIR Consortium (2013) Inhaled Colistin: A Novel Approach for Reducing Drug-Resistant TB Transmission. Abstract presented at the 17th Annual Conference of the Union – North America Region, Vancouver, Canada, 28th February to 2nd March 2013.

[76] Dharmadhikari, A.S. and Nardell, E.A. (2008) What animal models teach humans about tuberculosis. *American Journal of Respiratory Cell and Molecular Biology*, **39**(5), 503–508. PubMed PMID: 18556589.

[77] Choudhary, D., Hegde, P., Voznesensky, O. *et al*. (2015) Increased expression of L-selectin (CD62L) in high-grade urothelial carcinoma: A potential marker for metastatic disease. *Urologic Oncology*, **33**(9), 387 e17–e27. PubMed PMID: 25618296. Pubmed Central PMCID: 4510033.

[78] Pham, D.D., Fattal, E. and Tsapis, N. (2015) Pulmonary drug delivery systems for tuberculosis treatment. *International Journal of Pharmaceutics*, **478**(2), 517–529. PubMed PMID: 25499020.

[79] Dutt, M. and Khuller, G.K. (2001) Chemotherapy of Mycobacterium tuberculosis infections in mice with a combination of isoniazid and rifampicin entrapped in Poly (DL-lactide-co-glycolide) microparticles. *Journal of Antimicrobial Chemotherapy*, **47**(6), 829–835. PubMed PMID: 11389115.

[80] Swaminathan, S. and Rekha, B. (2010) Pediatric tuberculosis: global overview and challenges. *Clinical Infectious Diseases*, **50**(Suppl. 3), S184–S194. PubMed PMID: 20397947.

[81] McIlleron, H., Willemse, M., Werely, C.J. *et al*. (2009) Isoniazid plasma concentrations in a cohort of South African children with tuberculosis: implications for international pediatric dosing guidelines. *Clinical Infectious Diseases*, **48**(11), 1547–1553. PubMed PMID: 19392636.

[82] Burman, W.J. and Jones, B.E. (2001) Treatment of HIV-related tuberculosis in the era of effective antiretroviral therapy. *American Journal of Respiratory and Critical Care Medicine*, **164**(1), 7–12. PubMed PMID: 11435232.

[83] Donald, P.R. (2007) The assessment of new anti-tuberculosis drugs for a paediatric indication. *International Journal of Tuberculosis and Lung Disease*, **11**(11), 1162–1165. PubMed PMID: 17958976.

[84] Furin, J., Brigden, G., Lessem, E. and Becerra, M.C. (2013) Novel pediatric delivery systems for second-line anti-tuberculosis medications: a case study. *International Journal of Tuberculosis and Lung Disease*, **17**(9), 1239–1241. PubMed PMID: 23827936.

20

Concluding Remarks: Prospects and Challenges for Advancing New Drug and Vaccine Delivery Systems into Clinical Application

P. Bernard Fourie[a] *and Richard Hafner*[b]

[a] *Department of Medical Microbiology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa*
[b] *Tuberculosis Clinical Research Branch, Division of AIDS, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA*

20.1 Introduction

Given the considerable advances over the past decade in pharmaceutical formulation and delivery technologies, and of an increasingly better understanding of host–pathogen interactions in tuberculosis (TB), lung-targeted drug and vaccine delivery systems to achieve enhanced prevention and treatment efficacy for *Mycobacterium tuberculosis* (*M. tuberculosis*) infection and disease have become viable options. In the foregoing chapters, authors highlight, for example, the desirability and feasibility of delivering anti-TB medications and vaccines to the lung and pulmonary cells/fluids using novel formulations of agents normally administered orally or parenterally.

A comprehensive review of inhaled-drug therapy for treatment of TB appeared in 2011 [1], highlighting the growing interest in the lung as a portal for anti-TB drug delivery. The many advantages for directly targeting the lung as the primary site of infection were emphasised.

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

Direct exposure of organisms infecting pulmonary macrophages or granuloma to high levels of drug is desirable. Cells in the lung, and in particular the alveolar space, might also be better suited to receive and process molecules with sub-optimal oral absorption and bioavailability or associated with substantial metabolism by hepatic first-pass metabolism, high levels of hepatic toxicity associated with first-pass metabolism, or toxicities caused by systemic exposure.

High extracellular lung fluid (ELF) levels are also important for some drugs, particularly for killing of extracellular bacilli and may also prevent emergence of drug resistance. This review laid the foundation for the contributions to this volume. Other areas being explored include micro- or nano-particle constructs for drug delivery to specific cells or organs; for example, targeted to phagocytes and allowing for release of drug only after cellular uptake. Significant progress has been made in recent years to develop appropriate formulations and delivery systems that may result in better efficacy. Likewise, live whole-cell, subunit or viral-vectored vaccines and adjuvants formulated as dry-powder aerosols or nanoparticle constructs hold promise. Delivery of vaccines to inducible bronchus-associated lymphoid tissues (iBALT) is a particularly promising new approach for TB [2]. Another future research area is delivery of host-directed therapies (HDT) for TB as treatment adjuncts to increase bacterial clearance and reduce inflammatory lung damage [3, 4]. Delivering corticosteroids (or other immunomodulators) to the lung to decrease pulmonary inflammation and avoid systemic adverse events serves as an example.

Unfortunately, to a certain extent, all of these delivery approaches have constraints to their successful translation to efficacious and safe therapeutic options. Moving the field forward adequately will depend on a clear understanding of how novel delivery systems may address current and emerging needs for improved tuberculosis clinical management, and prevention of infection, disease, and drug resistance. From the accounts delivered by authors in this volume, significant advances clearly have been made towards defining appropriate new interventions based on innovative drug and vaccine delivery systems. Some gaps in the development pipeline are better understood, and a roadmap is emerging that will hopefully enable development barriers to be overcome.

Many challenges have not been fully met, or addressed at all. Some of the remaining major issues include accurate quantification of dose deposition and distribution following pulmonary delivery in humans. In particular, adequate delivery and safety of inhaled drugs into diseased and damaged airways/tissues, as in the case of tubercular cavities and necrotic, badly ventilated areas of the lung, need to be defined. Ensuring adequate drug levels inside the granuloma is, likewise, an important objective.

20.2 Progress in the Formulation and Manufacturing of Drugs and Vaccines for Tuberculosis

20.2.1 Inhaled Drugs and Drug Combinations

The viability of pulmonary administration of anti-TB therapy depends on the successful formulation of drugs into powders or other forms to allow deposition of drug particles in the alveolar regions of the lung. For success in the operational setting, the use of simple inhaler devices for inhaled delivery of anti-TB drugs will be critical.

Micro- and nano-particle constructs for a variety of TB drugs have been successfully developed and reported, potentially providing for deep penetration into airways, rapid uptake by alveolar macrophages, and reaching very high levels in lower airway/alveolar extracellular fluids. However, effective delivery remains challenging. Makino and Terada [5] have identified the tendency for nanoparticle agglomeration as a potential barrier to delivery to reaching the alveolar space, whilst unaggregated particles are exhaled due to their low inertia. Large porous microparticles in the 2–4 micron diameter range resulting from spray-drying allow for the formulation of inhalable drug powders that overcome these constraints; that is, small aerodynamic size despite larger surface area that ensures improved delivery to the alveolar space. Das and Stewart [6] further elaborate on techniques to overcome physical constraints in particle delivery, including the use of appropriate devices.

The use of pharmaceutical excipients to act as carriers for micro- /nano-particles generally improves the delivery characteristics. Recent developments include rifampicin–lactose microparticle composites prepared by a supercritical antisolvent drug excipient mixing technique which would likely result in microparticles in the 2–4 micron range suitable for inhalation. Other techniques to generate particles to improve solubility of drugs with poor bioavailability include nanosuspensions and microliposomes. In both cases, administration would be by the oral or parenteral routes because of the need for liquid carriers or complicated devices for administration as aerosols.

Several recent studies have examined the potential use of known injectable or orally administered anti-TB agents as spray-dried powder formulations shown in animal and human studies to be highly suitable for pulmonary administration. Capreomycin [7], rifapentine [8], pyrazinoic acid [9], and clofazimine [10, 11] show promise as candidates for formulation into inhaled dosage forms. Capreomycin has already been administered to human subjects as an inhalable agent [7]. Inhaled delivery of pyrazinoic acid, the active moiety of pyrazinamide, could hold promise for enhanced efficacy and for potentially overcoming drug resistance conferred by mutations in the *pncA* gene [9]. Clofazimine (CFM) might benefit from pulmonary rather than oral administration since avoidance of skin discoloration or gastric intolerance as major adverse effects might be achieved [10]. CFM, a lipophilic riminophenazine antibiotic, possesses both antimycobacterial and anti-inflammatory activity. Inhalable CFM-containing dry-powder microparticles and native CFM were evaluated for activity against *M. tuberculosis* in human monocyte-derived macrophage cultures and in mice infected with a low-dose aerosol. Spray-dried clofazimine was suitable for deep lung delivery, retained the *in vitro* kill characteristics of the native compound, and demonstrated efficacy in preliminary *in vivo* experiments [10, 11].

Misra and colleagues [12] comment favourably on the utility of several excipient-free drug combinations prepared as spray-dried powders for inhalation, including rifapentine–moxifloxacin–pyrazinamide and rifampicin–isoniazid–pyrazinamide combinations [13, 14]. The utility of first-line oral replacement therapies by inhaled options is debatable, if potential treatment shortening or improved cure is the ultimate aim. No evidence is available that such advantages, or better compliance with drug-taking, will result from changing the route of administration of these drug combinations for drug-sensitive TB. The most feasible application of inhaled-drug treatment is in the context of adjunct therapies to conventional oral/parenteral drug combinations to more aggressively reduce bacterial burden.

Buttini and Colombo [15] and Young *et al*. [16] summarised the recent experience with manufacture and delivery of powders for inhalation and liquids for nebulisation. The advantages of spray-dried powders over nebulised formulations lie in operational feasibility in high-burden settings, as well as favourable pharmaceutical properties that allow for efficient deposition in the alveolar region of the lung. In addition, up to 25 mg of active drug ingredient can be filled into capsules for use in simple breath-activated handheld inhalers. Spray-dried powders generally also show good shelf-stability at ambient temperatures [15]. Liquid formulations for inhalation are usually more difficult to prepare as stable solutions, require a significant quantity of liquid carrier, pose greater challenges for delivery devices (e.g., nebulisers), and consequently might be more costly to produce at scale.

Wong *et al*. [17] expand on the usefulness of spray-drying anti-TB drugs into inhalable powders and present a complete listing of formulations successfully spray-dried between 1999 and 2014 [17]. Almost every known anti-TB drug, including some new agents, has been produced in this form. They point out, however, that translation into clinical practice is still lacking. A sound rationale around utilisation of inhaled drugs in treatment regimens has not been offered thus far, and a definition of clinical trial approaches to demonstrate utility in human subjects is lacking. These issues remain as major challenges toward translation of inhalable anti-TB agents for use in the clinical setting.

An innovative approach in drug formulation demanding attention is the concept of ion-pairing (the association of oppositely charged ions in solution to form distinct chemical species) [18]. Although the concept of ion-pairing is not new, its application to inhaled-drug formulation is recent, in particular as regards anti-TB drugs. Using this approach, Giovagnoli and colleagues [19] characterised novel palladium (Pd) complexes with second-line antitubercular drugs, namely capreomycin, kanamycin, and ofloxacin, and described their *in vitro* extracellular and intracellular activity against *M. tuberculosis* infection. The novel anti-TB complexes had extracellular activity comparable with that of free drugs and improved efficacy against *M. tuberculosis* intracellular infection.

Respirable bacteriophage formulations provide another perspective on innovative therapeutic options in the treatment of TB. This concept is in its infancy, but might be worthwhile pursuing. Hatfull and Vehring [20] recognise that mycobacteriophage therapy in humans has not been reported, and a clear identification of which phages (or non-replicating phage components) to select for the purpose has not been achieved. However, *M. smegmatis* as non-pathogenic surrogate infected with phage TM4 (other options are D29 from cluster A2, DS6A, or candidates from clusters G and K that do not show cross resistance to other phage choices) could theoretically be targeted to cells infected with *M. tuberculosis*. Seeding cells with mycobacteriophages should lead to a sharp reduction in intracellular pathogenic *M. tuberculosis.* Similar scenarios relate to the use of inducible lysogen that enters lytic growth in infected macrophages. Suitable respirable delivery options would also need to be developed, but should be feasible, given the progress with dry-powder preparations of Bacillus Calmette–Guérin (BCG) intended for delivery by small, inexpensive handheld inhalers [21]. Early work is limited, but exploration continues for these promising concepts [22–25]. The primary requirement is to ensure safety by proving that the surrogate bacterial host does not cause disease or adverse responses.

Turning attention to RNA nanoparticles as potential vaccines [26], only four RNA-based vaccines have so far progressed into clinical trials. None are directed at TB, two are targeted to human cytomegalovirus, one to stage IV renal carcinoma, and one to metastatic

melanoma [27–30]. Ensuring that stability is maintained and binding to the nanoparticle carrier is achieved are necessary in manufacturing RNA for cell delivery to elicit appropriate T-cell responses (priming T-helper cells and dendritic cell activity). These are key challenges. RNA aptamers as potential cell- and tissue-targeting agents with potential for enhancing protection against TB have been discussed and experimentally assessed with promising results in mice and monkeys [31]. Possible lung delivery applications have also been considered [32]. Multi-functional RNA nanoparticles which contain toll-like receptor agonist RNA molecules or aptamers mediating delivery of chemotherapeutic drugs to T cells may also be created [33–35]. Poly(inosinic:cytidylic acid) (poly I:C) co-incorporated in nanoparticles may potentiate antigen presentation [36] and are potentially amenable to TB vaccination.

Subunit-vectored vaccines are also being investigated for use in TB. As elaborated upon in Chapter 5 [37], a recent study of the TB vaccine candidate MVA85A in non-human primates (NHPs) investigated the safety and immunogenicity of the vaccine delivered directly to the lungs of the animals, using a nebuliser approved for human use, vs. vaccine delivered by intradermal injection [38]. The study detected no adverse effects to any route of delivery. Importantly, the experiment demonstrated feasibility in a model that mimics potential human administration closely. Human trials to evaluate the route of administration and correlations with induced immune responses are underway [39].

20.3 Considerations in the Development of TB Drug and Vaccine Delivery Options

20.3.1 Lung Biology and Pulmonary Administration of Drugs and Vaccines

Mechanisms that regulate the uptake of *M. tuberculosis* into alveolar macrophages after inhalation are likely to be also important for ensuring successful vaccination with live, whole-cell mycobacterial vaccines delivered to the lung, such as BCG, recombinant forms of BCG, or attenuated *M. tuberculosis*. Effectively mobilising respiratory immune cells, such as dendritic cells, as the key initiators of the immune response [40, 41], and to a lesser extent perhaps also neutrophils [42], would be key considerations in the presentation of live vaccine in order to elicit adequate immune responses. A better understanding of the role of dendritic cell subsets and the regulators of their interactions with T cells in eliciting immune responses is, however, required. A new attenuated *M. tuberculosis* vaccine administered to macaques by inhalation has shown promise, pointing to activation of iBALT as a requirement [2, 43]. Recently, the considerable functional diversity of mucosal-associated invariant T (MAIT) cell responses was demonstrated in mice, as well as that nonpolymorphic major histocompatibility complex class I-like molecule (MR1)-restricted MAIT cells are important for TB protective immunity [44].

Furthermore, the capacity of membranous epithelial, microfold or microvillous cells (M cells) to sample antigens, transfer luminal bacteria, and stimulate mucosal immune responses, makes them fascinating targets for mucosal vaccine delivery [45]. Results of previous studies imply that safe and effective oral mucosal vaccines may require improved M cell-targeting strategies, because the M cell-targeting vaccines now available do not actively direct antigens to M cells. Recent studies have searched for specific molecules to target M cells, for improvement of antigen processing and presentation. However, many

mechanisms of M cells are still unclear and are not as well known as those of dendritic cells (DCs). Therefore, to develop M cell-targeting strategies in mucosal vaccines, further understanding of mucosal immunology and M cell biology is required. Knowledge of specific components and regulation of M cell function and identification of more effective M cell-specific molecules are crucial for further development of M cell-targeted vaccines [45].

In the context of drug therapy, two key first-line drugs used in the treatment of TB, isoniazid and rifampicin, have been shown to induce autophagy in *M. tuberculosis*-infected macrophages that more effectively inhibit organism growth [46]. Targeting autophagic function is being investigated as a treatment option for TB following observations *in vivo* that autophagy-deficient mice show increased *M. tuberculosis* growth and lung pathology, and reduced survival when compared with normal mice [47–49]. Autophagy inducers like rapamycin delivered by inhalation may be highly useful to induce autophagy in infected cells and to avoid systemic side effects.

Muttil and Price [50] provide some thoughtful insights on how natural immunity might be harnessed via pulmonary administration of TB vaccines to enhance efficacy. They argue that the use of attenuated bacterial strains for immunisation that resemble the infecting organism as closely as possible, including its natural route of transmission and exposure, may be essential for generating a robust and long-lived immunity against TB. Importantly, recent (unpublished) work from the Muttil laboratory lends further support to the need for exploration of the pulmonary route for vaccination against TB. Individuals living in areas with high prevalence of environmental non-tuberculous mycobacteria (NTM) are known to have high rates of low-level tuberculin skin test sensitivity unrelated to TB infection. Mice exposed to NTMs via the gastrointestinal tract (mimicking NTM exposure in humans) became immunologically tolerant or non-responsive to mycobacterial exposure, including BCG. Of great significance is the observation that this tolerance effect appears to be systemically widespread, the immunologically naïve airway remains unaffected. In a challenge model of mice exposed to NTM, only pulmonary-vaccinated mice were able to generate immune cells in the airway and had a log less *M. tuberculosis* colony-forming units (CFUs) in the lung compared with intradermally vaccinated NTM-exposed mice.

A growing interest in exploring mucosal delivery for TB vaccines has to some extent benefitted from major advances in aerosol technology over the last 10–15 years. More effective, reproducible, and inexpensive delivery of proteins and vaccines delivered via the pulmonary route has shown equivalent or better efficacy for encephalitis, hepatitis B, plague, measles, and influenza prevention. Applying advanced aerosol science and technology to the search for a new and more effective TB vaccine may improve the chances – and speed – of clinical success. Critical challenges relate to process, formulation, and delivery approaches, particularly for infant vaccination, however.

20.3.2 Choice of Animal Model in the Evaluation of Drug and Vaccine Delivery Systems

As the complexities of lung pathology are increasingly better understood and advances in molecular biology and imaging techniques move forward, the limitations of the traditional mouse and guinea-pig models for investigation of drug and vaccine efficacy have become clear. Ideally, research should be expanded to involve other species (or alternatively utilise current species) in order to accommodate more advanced technologies in determining

outcomes. One key question is to determine which animal models might provide the appropriate pre-clinical information to assess potential efficacy of candidate drugs and vaccines administered by the pulmonary route and of orally administered vaccines.

Validated immunological markers of protection in TB vaccine assessment cannot be extrapolated from animals to humans [51–54]. The inability to select candidate vaccines in a head-to-head strategy in the same animal model, mostly as a result of the reluctance of vaccine developers to have their candidates enter comparative testing, is a principal weakness in TB vaccine development [52, 53]. Even so, current animal models and assays for vaccine assessment seem to be inadequate, given the failure of MVA85A vaccine to boost immune responses in BCG-vaccinated infants, although pre-clinical data from animal studies predicted otherwise [51]. NHPs are increasingly being used as higher-order models for evaluating TB vaccine candidates. In this context, Rhesus and Cynomolgus macaques have been utilised as models of vaccine efficacy, because both produce human-like pathology when infected with *M. tuberculosis* [55, 56]. Recently, the common marmoset was reported to develop a very human-like disease following low-dose, aerogenic infection with several strains of *M. tuberculosis*. Given their small size and ease of handling relative to macaques, they might become established as a new animal model for assessing vaccines in the near future [57]. Also, a recent description of a neonatal Rhesus macaque model that mimicked human TB in new-borns and infants might open the way for specific assessment of disease prevention by therapeutic vaccines in this age cohort [58].

For drug therapy assessment, on the other hand, conventional mouse models seem to be more dependable as an essential pre-step to drug development [59–61].

Emerging animal models contributing to our understanding of the pathogenesis of pulmonary TB include the rabbit (not new, but neglected for decades). Other species, such as the rat, zebrafish, and wildlife reservoirs have also been investigated, but their roles remain undefined in the pre-clinical development of drugs and vaccines for TB. The most encouraging developments are the recent improvements to the established mouse, guinea-pig, rabbit and NHP models, including the application of new imaging technologies in mice, rabbits and NHPs to assess changes in lung pathology. Positron-emission tomography/computerised tomography (PET/CT) imaging is being explored to describe the effect of drug or vaccine interventions on TB pathogenesis and to study the effect of therapeutics on inbred mouse strains that produce hypoxic, necrotic TB granulomas, similar to human TB disease. “Kramnik mice” are a recently introduced mouse model developing granulomas/necrotic areas against a genetically immunocompromised background. Compared with the usual mouse models, they are not better than Balb-c mice for drug evaluation, but can give different results. A promising, but significantly under-utilised, application of PET/CT imaging is its potential use in studying pulmonary distribution and quantification of pharmacokinetic/pharmacodynamics (PK/PD) characteristics of inhaled drugs. Such data would go a long way towards providing evidence of appropriate drug deposition at target sites.

20.3.3 Demonstrating Bioequivalence and Clinical Efficacy of Inhaled Drugs to Oral/Parenteral Dosage Forms

In drug development and clinical assessment of drug efficacy, the pharmacokinetics profile of the active ingredient is crucially important when preparing a product profile for licensing consideration by regulatory authorities. The most notable feature of inhaled-drug

delivery is the fact that a high concentration of drug may be deposited in the airways, ideally with short residence times (determined by particle formulation) during which time drug is dissolved in lung alveolar fluid, possibly diffusing across the epithelium and being phagocytosed by alveolar and tissue macrophages. In this way, drug distribution into pulmonary tissues could also occur at non-ventilated sites.

Only a limited amount of drug will normally end up in the systemic circulation, posing a dilemma for measuring serum levels and consequently determining bioequivalence to oral preparations containing the same drug. This aspect poses a barrier to regulatory assessment and approval of the inhaled-drug dosage form. In studies of dry-powder microparticle preparations of capreomycin delivered to the lungs of guinea-pigs, compared with the conventional intramuscular injection route, 30% higher drug levels were required by the injected route relative to the inhalation route to achieve a similar 1 log drop in lung CFU of infected animals [62]. The differences in pharmacodynamics effect were significantly in favour of the inhalation route, suggesting that lowering of capreomycin dosage might be possible when delivered directly to the lung. In follow-up healthy human volunteer Phase I studies with the same dry-powder capreomycin preparation, a 300 mg inhaled dose achieved peak systemic levels that briefly reached above the MIC of the drug [7]. In Chapter 8 [63], the authors compared the guinea-pig [62] and human data [7] and observed that (a) the highest dose given to humans (3.77 mg/kg) was in between the low and high doses given to guinea-pigs (1.4 and 7.2 mg/kg); (b) the dose in humans resulted in approximately a similar maximum drug concentration (C_{max}) (2.14 µg/ml / 3.77 mg/kg) to that in guinea-pigs when corrected by dose (3.34 µg/ml / 7.2 mg/kg); that (c) the time to maximum drug concentration (T_{max}) was about 7 times longer in humans than in guinea-pigs (2.8 h versus 0.38 h) and the half-life $T_{1/2}$ was almost 3 times as long (4.8 h in humans versus 1.68 h in guinea-pigs); and that (d) apart from obvious large differences in body size, other factors that might have played a role are passive inhalation versus insufflations, the alveolar area available for dissolution, and differences in the metabolic rate between these two species.

From a feasibility point of view, this dose is probably the highest that could be delivered as a single administration. Although bactericidal levels at this dose, and perhaps even at 150 mg, are likely achieved in the lung as extrapolated from the guinea-pig data, regulators would probably remain unconvinced unless strong clinical PK/PD data are shown.

A bioequivalence model is required that would provide for the direct comparison of inhaled drug to drug delivered in oral/parenteral form, with a bacteriological outcome as primary endpoint. Demonstrating the efficacy of inhaled dosage forms of TB drugs in developmental programmes would likely only be achieved by using a combination of mouse and guinea-pig pre-clinical assessment, which would include the collection of PK data at this level of investigation (Chapter 8), followed by early bactericidal activity (EBA) studies in Phase II clinical trials. Considerable experience in animal models has been gained over recent years with demonstrating the superiority of the pulmonary route of administration of certain TB drugs over oral or parenteral delivery, both in terms of efficacy and also of safety. In the process, adequate animal models for comprehensive PK/PD assessment have been established that can serve as validation assays for the pre-clinical phase of inhaled-drug development [41, 63].

What remains to be achieved is the demonstration of a valid clinical trial approach that can be used to assess outcomes among patients with TB. Furthermore, until such time as a well-constructed clinical-development strategy is proposed for the introduction of inhaled

agents as an alternative to oral administration of established drugs in first- or second-line regimens for TB, inhaled-drug therapies of known drugs might only be acceptable as adjunct options to conventional drug regimens, not as replacements.

EBA studies have been widely used in TB drug development as supporting information to medicines-regulatory authorities. However, most of the drugs that would be priority candidates for pulmonary delivery as argued earlier, such as the injectable agents capreomycin or kanamycin, or the second-line drug clofazimine, are known to have substantial sterilising rather than early bactericidal activity. With a typical EBA study running over the first 14 days of therapy, this might not be the ideal approach for evaluating the drugs in question. Furthermore, high drug levels in the upper airways might compromise measurements of viable colonies in sputum collected but not processed instantaneously. An extended EBA design providing for sputum collections over 8 to 12 weeks with timed drug wash-out periods might be an option, but has not been yet tried. Even so, early sputum conversion does not necessarily indicate a lowered risk for relapse or serve as an indicator of potential treatment shortening, as was observed in the recently concluded REMox trial [64]. The only option might be to conduct a conventional EBA study to assess safety profiles, followed by a Phase II proof-of-culture-based study, and then a larger Phase III clinical endpoint study of inhaled adjunct therapy added to a standardised backbone regimen against placebo in a double-blind trial design over a full course of treatment in drug-sensitive and/or multi-drug-resistant TB (MDR-TB) patients.

20.3.4 Inhaled Vaccines for TB – are there Potential Advantages?

Natural infection with tubercle bacilli almost exclusively occurs following inhalation of droplet nuclei containing live organisms transmitted by patients with pulmonary TB disease. In well over 90% of exposed individuals, the infection is contained indefinitely as a result of a sufficient cell-mediated immune response in the host. In those who develop active disease, however, the process is influenced by a variety of factors related to the host and/or the pathogen. Most cases develop within two years after the point of exposure, with a life-time risk of 5–10%. The risk for disease is particularly high in infants and young children, with extrapulmonary pathology (tuberculous meningitis, in particular) often leading to disability and death. Immunocompromised individuals, such as those living with HIV, are at significantly increased risk of progressing to active TB. The risk changes to 10% per year, rather than remaining at that level over a life-time. Other high-risk groups include adolescents and a high-risk group only very recently identified, Type 2 diabetics. About 387 million people are estimated to be affected by diabetes, with 77% of cases in low- and middle-income countries where TB is prevalent. In the light of studies showing that between 16% and 46% of TB cases also suffer from diabetes, the World Health Organization (WHO) has turned the spotlight on this group as a priority for intervention [65].

Overall, it would seem that the lung is immunologically geared to effectively deal with TB infection in most cases. In this context, targeting vaccines for TB, particularly live, whole-cell candidates, directly to the lung is a logical approach.

In Chapter 5, a comprehensive overview of the status of TB vaccine development is provided. For many of these candidates, administration to the lung rather than by injection into the skin would be possible, and at least one such study is currently in progress [53].

Further exploration of dry-powder pulmonary administration of BCG, currently still the only licensed TB vaccine for use in humans, has been advocated [66].

Most importantly, delivery to the lung may provide efficacy at significantly lower body dose, as suggested from (uncontrolled) results obtained in the early 1960s for aerosolised liquid BCG vaccine relative to intradermal injection. In these studies, using various types of nebulisers and chambers to generate aerosols with predictable concentrations of BCG and exposures up to 30 minutes, about 10,000 CFU (as opposed to $2–8 \times 10^{-5}$ CFU for intradermal injection of, e.g., BCG Danish strain) resulted in a high degree of low-dose tuberculin skin-test conversion retained in children for up to 4 years after vaccination. No cases of TB were diagnosed among vaccinated children during the ensuing decade [67, 68]. From these observations (uncontrolled), Rosenthal concluded that '*… Aerosol vaccination could be applicable for mass vaccination. A mobile console that would automatically disperse the vaccine … would facilitate the procedure.*' [68].

20.3.5 Safety of Pulmonary Vaccination

Pulmonary immunisation against TB has been evaluated for feasibility and efficacy in animal models and has shown that better protection is afforded compared with immunisation by intradermal injection of Copenhagen strain SSI 1331 [22]. It is not known whether this observation holds true for all BCG strain types is unknown. However, pulmonary immunisation against TB has not been able to cross regulatory hurdles due to the potential risk associated with this delivery route. Encouragingly, a recent Phase I safety and immunogenicity study was undertaken in BCG-vaccinated healthy adults with the MVA85A vaccine delivered as an aerosol [39]. Vaccination by the pulmonary route was well tolerated, with only mild respiratory adverse events recorded.

Development of effective and safe adjuvants that could be delivered by the pulmonary route is also a priority. Very few adjuvants are approved for human use and none of them for pulmonary delivery. The adjuvants used for parenteral vaccines, including alum, are not safe for pulmonary delivery [69]. Muramyl dipeptide (MDP) and trehalose dibehenate (TDB) were evaluated in *in vitro* studies for use as an adjuvant in inhaled TB vaccines [70]. However, many pulmonary immunisation studies use vaccines that have inherent adjuvant properties, including live bacterial and viral vector-based vaccines. New concepts for vaccine potentiation by co-administration of agents to modify functions of relevant immune cells are also in evaluation, including immunomodulation strategies to enhance CD8 T cell responses to antigens by overcoming immunosuppressive microenvironments. For example, inhibiting mammalian target of rapamycin (mTOR) in antigen-specific CD8 T cells during acute infection or vaccination can enhance generation of memory CD8 T cells with improved quality characterised by high proliferative and protective capacity and increased longevity. Such innovative approaches may be adapted in development of inhaled vaccines that could be co-formulated with such potentiating agents [71, 72].

Delivering a live vaccine by the pulmonary route, however, could lead to dissemination and disease, especially in immunocompromised individuals. However, different doses of live BCG administered by the pulmonary route in cancer patients are safe and without any major side effects [73]. BCG was well tolerated in metastatic lung cancer patients, with symptoms consisting of transient chills for 4–8 hours post-BCG aerosol delivery.

In addition, rhesus macaques were vaccinated by the pulmonary route with a replication-defective recombinant adenovirus (rAd; AERAS-402) expressing the Mtb antigens Ag85A and TB10.4 [74]. The AERAS rAd is a safe vaccine-delivery vector due to its non-replicating nature and was suited for pulmonary immunisation due to its natural tropism for the respiratory tract [75].

20.4 Concluding Remarks

Development of innovative new strategies for pulmonary delivery of antimicrobial drugs, host-directed therapies, and vaccines/adjuvants is hindered by a lack of research support. Progress could be made by building on the large and detailed amount of research to produce sophisticated formulations and devices to facilitate delivery of drugs and vaccines via the pulmonary route for other diseases. A critical need exists for devising and implementing clinical strategies and proof-of-concept trial designs to evaluate conventional and new therapies directed to the lung, including drugs added to accepted regimens and novel regimens aimed at shortening MDR-TB treatment.

What key innovations need to be considered other than optimised formulations and delivery devices? At the high level, the following might serve as a starting list:

- Developing combinations of anti-TB and/or host-directed therapy drugs: Evaluation of a carefully considered "best-shot" inhaled regimen for proof-of-concept studies should be pursued;
- Utilizing a robust animal model to optimise inhaled TB drugs/regimens or HDT agents, including dose, schedule, and duration for shortened therapy for MDR-TB (and perhaps also for susceptible TB) proof-of-concept would be to demonstrate lack of relapse with the shortened therapy vs. control;
- Evaluating pulmonary distribution of labelled drug by PET/CT imaging, first in animals and later in humans;
- Clinical Trials: Phase IIB trials to evaluate the effects of addition of inhaled agent(s) or placebo to a conventional oral drug regimen on rates of CFU decline in sputum collected bi-weekly over 8–12 weeks. To avoid confounding effects of high inhaled drug concentrations in upper airways, a 2-day wash-out before sputum collection may be needed. Alternatively, methods to be developed to immediately deactivate any remaining drug in collected sputum without affecting *M. tuberculosis* viability. Also, use of PET-CT imaging for evaluation of treatment responses might be employed and its utility defined as a marker of response. If results are promising, a Phase III trial based on clinical endpoints, including failure/relapse rates would follow.

The contributions shared in this volume demonstrate a dynamic and progressively expanding portfolio in the field of drug delivery system development for TB. In all cases, the objectives are to enhance efficacy and possibly reduce occurrence of adverse events by optimising drug and vaccine delivery options. Technologies already exist for formulation and manufacturing of, for example, nano- and micro-particle constructs that may be used as drug and vaccine carriers for targeted delivery to cells using appropriate delivery devices. The potential clinical benefits of these developments can be demonstrated in a growing array of applicable animal models, and to a large extent have already informed study

designs for clinical trials. However, the translation of research advances from the laboratory to clinical implementation is lacking and needs to be pursued as a matter of high priority. In this context, regulatory experience with inhaled and other alternative means of administration of therapies and vaccines for TB is almost non-existent; the development of guidelines for review and approvals will require close interaction with scientists and developers. Policies to guide implementation in clinical settings would also need to be developed, with involvement of national TB programmes and international agencies such as the WHO. Without support for such efforts, the field will, unfortunately, not progress beyond the status quo.

References

[1] Misra, A., Hickey, A.J., Rossi, C. *et al*. (2011) Inhaled drug therapy for treatment of tuberculosis. *Tuberculosis (Edinburgh)*, **91**(1), 71–81. Epub 2010 Sep 27.

[2] Kaushal, D., Foreman, T.W., Gautam, U.S. *et al.* (2015) Mucosal vaccination with attenuated *Mycobacterium tuberculosis* induces strong central memory responses and protects against tuberculosis. *Nature Communications*, **6**, article 8533.

[3] Mahon, R.N. and Hafner, R. (2015) Immune cell regulatory pathways unexplored as host-directed therapeutic targets for *Mycobacterium tuberculosis*: An opportunity to apply precision medicine innovations to infectious diseases. *Clinical Infectious Diseases*, **61**(Supplement 3), S200–S216.

[4] Wallis, R.S. and Hafner, R. (2015) Host-directed therapies for tuberculosis. *Nature Reviews Immunology*, **15**(4), 255–263.

[5] Makino, K. and Terada, H. Chapter 8 (this volume).

[6] Das, S.C. and Stewart, P.J. Chapter 13 (this volume)

[7] Dharmadhikari, A.S., Kabadi, M., Gerety, B. *et al.* (2013) Phase I, single-dose, dose-escalating study of inhaled dry powder capreomycin: A new approach to therapy of drug-resistant tuberculosis. *Antimicrobial Agents and Chemotherapy*, **57**(6), 2613–2619.

[8] Chan, J.G., Bai, X. and Traini, D. (2014) An update on the use of rifapentine for tuberculosis therapy. *Expert Opinion on Drug Delivery*, **11**(3), 421–431.

[9] Mitchison, D.A. and Fourie, P.B. (2010) The near future: Improving the activity of rifamycins and pyrazinamide. *Tuberculosis*, **90**(3), 177–181.

[10] Cholo, M.C., Steel, H.C., Fourie, P.B. *et al*. (2011) Clofazimine: Current status and future prospects. *Journal of Antimicrobial Chemotherapy*, **67**(2), 290–298.

[11] Verma, R.K., Germishuizen, W.A., Motheo, M. *et al*. (2013) Inhaled microparticles containing clofazimine are efficacious in the treatment of experimental tuberculosis in mice. *Antimicrobial Agents and Chemotherapy*, **57**(2), 1050–1052.

[12] Misra, A. *et al.* Chapter 11 (this volume).

[13] Chan, J.G., Tyne, A.S., Pang, A. *et al*. (2014) A rifapentine-containing inhaled triple antibiotic formulation for rapid treatment of tubercular infection. *Pharmaceutical Research*, **31**(5), 1239–1253.

[14] Katiyar, S.K., Bihari, S. and Prakash, S. (2008) Low-dose inhaled versus standard dose oral form of anti-tubercular drugs: Concentrations in bronchial epithelial lining fluid, alveolar macrophage and serum. *Journal of Postgradraduate Medicine*, **54**(3), 245–246.

[15] Buttini, F. and Colombo, G. Chapter 10 (this volume).

[16] Young, E.F. *et al*. Chapter 6 (this volume).

[17] Wong, J. *et al*. Chapter 9 (this volume).

[18] Giovagnoli, S. *et al*. Chapter 12 (this volume).

[19] Giovagnoli, S., Marenzoni, M.L., Nocchetti, N. *et al*. (2014) Synthesis, characterization and in vitro extracellular and intracellular activity against *Mycobacterium tuberculosis* infection of new second-line antitubercular drug-palladium complexes. *Journal of Pharmacy and Pharmacolology*, **66**(1), 106–121.

[20] Hatfull, G.F. and Vehring, R. Chapter 14 (this volume).
[21] Garcia-Contreras, L., Wong, Y.L., Muttil, P. *et al*. (2008) Immunization by a bacterial aerosol. *Proceedings of the National Academy of Sciences of the USA*, **105**(12), 4656–4660.
[22] Liu, K., Wen, Z., Li, N. *et al*. (2012) The impact of relative humidity and collection media on mycobacteriophage D29 aerosol. *Applied Environmental Microbiology*, **78**(5), 1466–1472.
[23] Hoe, S., Semler, D.D., Goudie, A.D. *et al*. (2013) Respirable bacteriophages for the treatment of bacterial lung infections. *Journal of Aerosol Medicine and Pulmonary Drug Delivery*, **26**(6), 317–335.
[24] Hatfull, G.F. (2014) Mycobacteriophages: windows into tuberculosis. *PLoS Pathogens*, **10**(3), e1003953.
[25] Danelishvili, L., Young, L.S. and Bermudez, L.E. (2006) In vivo efficacy of phage therapy for *Mycobacterium avium* infection as delivered by a nonvirulent mycobacterium. *Microbial Drug Resistance*, **12**(1), 1–6.
[26] DeLong, R. Chapter 15 (this volume).
[27] Weide, B., Carralot, J.P., Reese, A. *et al*. (2008) Results of the first phase I/II clinical vaccination trial with direct injection of mRNA. *Journal of Immunotherapy*, **31**(2), 180–188.
[28] Rittig, S.M., Haentschel, M., Weimer, K.J. *et al*. (2011) Intradermal vaccinations with RNA coding for TAA generate $CD8^+$ and $CD4^+$ immune responses and induce clinical benefit in vaccinated patients. *Molecular Therapy*, **19**(5), 990–999.
[29] Bernstein, D.I., Reap, E.A., Katen, K. *et al*. (2009) Randomized, double-blind, phase 1 trial of an alphavirus replicon vaccine for cytomegalovirus in CMV seronegative adult volunteers. *Vaccine*, **28**(2), 484–493.
[30] Van Craenenbroeck, A., Smits, E., Anguille, S. *et al*. (2015) Induction of cytomegalovirus-specific T cell responses in healthy volunteers and allogeneic stem cell recipients using vaccination with messenger RNA-transfected dendritic cells. *Transplantation*, **99**(1), 120–127.
[31] Pan, Q., Wang, Q., Sun, X. *et al*. (2014) Aptamer against mannose-capped lipoarabinomannan inhibits virulent *Mycobacterium tuberculosis* infection in mice and rhesus monkeys. *Molecular Therapy*, **22**(5), 940–951.
[32] Resaee, F., Meednu, N., Emo, J.A. *et al*. (2011) Polyinosinic:polycytidylic acid induces protein kinase D-dependent disassembly of apical junctions and barrier dysfunction in airway epithelial cells. *Journal of Allergy and Clinical Immunology*, **128**(6), 1216–1224.
[33] Li, X., Zhao, Q. and Qui, L. (2013) Smart ligand: aptamer-mediated targeted delivery of chemotherapeutic drugs and siRNA for cancer therapy. *Journal of Controlled Release*, **171**(2), 152–162.
[34] Herrmann, A., Priceman, S.J., Kujawski, M. *et al*. (2014) CTLA4 aptamer delivers STAT3 siRNA to tumor-associated and malignant T cells. *Journal of Clinical Investigation*, **124**(7), 2977–2987.
[35] Lee, Y.R., Lee, Y.H., Im, S.A. *et al*. (2010) Biodegradable Hilleman, H. (2003) Critical overview and outlook: pathogenesis, prevention, and treatment of hepatitis and hepatocarcinoma caused by hepatitis B virus. *Vaccine*, **21**(32), 4626–4649.
[36] Lee, Y.R., Lee, Y.H., Im, S.A. *et al*. (2010) Biodegradable nanoparticles containing TLR3 or TLR9 agonists together with antigen enhance MHC-restricted presentation of the antigen. http://www.ncbi.nlm.nih.gov/pubmed/?term=tlr3+and+tlr9+arch+pharm. *Archives of Pharmaceutical Research*, **33**(11), 1859–1866.
[37] Loxton, A.G. *et al*. Chapter 5 (this volume).
[38] White, A.D., Sibley, L., Dennis, M.J. *et al*. (2013) Evaluation of the safety and immunogenicity of a candidate tuberculosis vaccine, MVA85A, delivered by aerosol to the lungs of macaques. *Clinical and Vaccine Immunology*, **20**(5), 663–672. PubMed PMID: 23446219. Pubmed Central PMCID: 3647747.
[39] Satti, I., Meyer, J., Harris, S.A. *et al*. (2014) Safety and immunogenicity of a candidate tuberculosis vaccine MVA85A delivered by aerosol in BCG-vaccinated healthy adults: a phase 1, double-blind, randomised controlled trial. *The Lancet Infectious Diseases*, **14**(10), 939–946. PubMed PMID: 25151225. Pubmed Central PMCID: 4178237.
[40] Kaufmann, S.H.E. and Schaible, U.E (2003) A Dangerous liaison between two major killers: *Mycobacterium tuberculosis* and HIV target dendritic cells through DC-SIGN. *Journal of Experimental Medicine*, **197**(1), 1–5.
[41] Kooyk, Y.V., Appelmelk, B. and Geijtenbeek, T.B.H. (2003) A fatal attraction: Mycobacterium tuberculosis and HIV-1 target DC-SIGN to escape immune surveillance. *Trends in Molecular Medicine*, **9**(4), 153–159.

[42] Lowe, D.M., Redford, P.S., Wilkinson, R.J. *et al*. (2012) Neutrophils in tuberculosis: friend or foe? *Trends in Immunology*, **33**(1), 14–25.
[43] Sakala, I.G., Kjer-Nielsen, L., Eickhoff, C.S. *et al*. (2015) Functional heterogeneity and anti-mycobacterial effects of mouse mucosal-associated invariant T cells specific for riboflavin metabolites. *Journal of Immunology*, **195**(2), 587–601. doi: 10.4049/jimmunol.1402545.
[44] Meierovics, A., Yankelevich, W.J. and Cowley, S.C. (2013) MAIT cells are critical for optimal mucosal immune responses during in vivo pulmonary bacterial infection. *Proceedings of the National Academy of Sciences of the USA*, **110**, E3119–3128.
[45] Wang, M., Gao, Z., Zhang, Z. *et al.* (2014) Roles of M cells in infection and mucosal vaccines. *Human Vaccines and Immunotherapy*, **10**(12), 3544–3551.
[46] Bento, C.F., Empadinhas, N. and Mendes, V. (2015) Autophagy in the fight against tuberculosis. *DNA Cell Biology*, **34**(4), 228–242.
[47] Stanley, S.A., Barczak, A.K., Silvis, M.R. *et al*. (2014) Identification of host-targeted small molecules that restrict intracellular *Mycobacterium tuberculosis* growth. *PLoS Pathogens*, **10**(2), e1003946. doi: 10.1371/journal.ppat.1003946.
[48] Hu, D., Wu, J., Xhang, R. *et al*. (2014) Autophagy-targeted vaccine of LC3–LpqH DNA and its protective immunity in a murine model of tuberculosis. *Vaccine*, **32**(20), 2308–2314.
[49] Olive, A.J. and Sassetti, C.M. (2014) New TB treatments hiding in plain sight. *EMBO Molecular Medicine*, **7**(2), 125–126.
[50] Muttil, P. and Price, D.N. Chapter 18 (this volume).
[51] McShane, H. and Williams, A. (2014) A review of preclinical animal models utilized for TB vaccine evaluation in the context of recent human efficacy data. *Tuberculosis (Edinburgh)*, **94**(2), 105–110.
[52] Barker, L., Hessel, L. and Walker, B. (2012) Rational approach to selection and clinical development of TB vaccine candidates. *Tuberculosis (Edinburgh)*, **92**(Supplement 1), S25–S29.
[53] Brennan, M.J. and Thole, J. (2012) Tuberculosis vaccines: a strategic blueprint for the next decade. *Tuberculosis (Edinburgh)*, **92**(Supplement 1), S6–S13.
[54] Principi, N. and Esposito, S. (2015) The present and future of tuberculosis vaccinations. *Tuberculosis (Edinburgh)*, **95**(1), 6–13.
[55] Scanga, C.A. and Flynn, J.L. (2014) Modeling tuberculosis in nonhuman primates. *Cold Spring Harbor Perspectives in Medicine*, **4**(12), a018564. doi:10.1101/cshperspect.a018564.
[56] Pena, J.C. and Ho, W.-Z. (2015) Monkey models of tuberculosis: lessons learned. *Infection and Immunity*, **83**(3), 852–862.
[57] Via, L.E., Weiner, D.M., Schimel, D. *et al.* (2013) Different virulence and disease progression following *Mycobacterium tuberculosis* complex infection of the common marmoset (*Callithrix jacchus*). *Infection and Immunity*, **81**(8), 2909–2919.
[58] Cepeda, M., Salas, M., Folwarczny, J. *et al*. (2013) Establishment of a neonatal rhesus macaque model to study *Mycobacterium tuberculosis* infection. *Tuberculosis (Edinburgh)*, **93**(Supplement 1), S51–S59.
[59] Lenaerts, A.J., DeGroote, M.A. and Orme, I.M. (2008) Preclinical testing of new drugs for tuberculosis: current challenges. *Trends in Microbiology*, **16**(2), 48–54.
[60] Nuermberger, E. (2008) Using animal models to develop new treatments for tuberculosis. *Seminars in Respiratory and Critical Care Medicine*, **29**(5), 542–551.
[61] Basaraba, R.J. (2008) Experimental tuberculosis: the role of comparative pathology in the discovery of improved tuberculosis treatment strategies. *Tuberculosis (Edinburgh)*, **88**(Supplement), S35–S47.
[62] Garcia-Contreras, L., Fiegel, J., Telko, M.J. *et al*. (2007) Inhaled large porous particles of capreomycin for treatment of tuberculosis in a guinea pig model. *Antimicrobial Agents and Chemotherapy*, **51**(8), 2830–2836.
[63] Ibrahim, M. and Garcia-Contreras, L. Chapter 7 (this volume).
[64] Gillespie, S.H., Crook, A.M., McHugh, T.D. *et al.* (2014) Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis. *New England Journal of Medicine*, **371**(17), 1577–1587.
[65] World Health Organization (2016) *Global Tuberculosis Report 2016*. World Health Organization, Geneva.

[66] Fourie, P.B., Germishuizen, W.A., Wong, Y.L. and Edwards, D.A. (2008) Spray drying TB vaccines for pulmonary administration. *Expert Opinion on Biological Therapy*, **8**(7), 857–863.
[67] Rosenthal, S.R., McEnery, J.T., Raisys, N. (1968) Aerogenic BCG vaccination against tuberculosis in animal and human subjects. *Journal of Asthma Research*, **5**(4), 309–323.
[68] Rosenthal R. (1980) Routes and methods of administration: Aerosol Vaccination, in *BCG Vaccine: Tuberculosis-Cancer* (ed. S.R. Rosenthal), PSG Publishing Company, Inc., Littleton, Massachusetts, pp. 170–172.
[69] Muttil, P., Prego, C., Garcia-Contreras, L. *et al*. (2010) Immunization of guinea pigs with novel hepatitis B antigen as nanoparticle aggregate powders administered by the pulmonary route. *AAPS Journal*, **12**(3), 330–337.
[70] Wang, C., Muttil, P., Lu, D. *et al*. (2009) Screening for potential adjuvants administered by the pulmonary route for tuberculosis vaccines. *AAPS Journal*, **11**(1), 139–147.
[71] Kamphorst, A.O., Araki, K. and Ahmed, R. (2015) Beyond adjuvants: immunomodulation strategies to enhance T cell immunity. *Vaccine*, **33** (Supplement 2), B21–B28.
[72] Araki, K., Turner, A.P., Shaffer, V.O. *et al*. (2009) mTOR regulates memory CD8 T-cell differentiation. *Nature*, **460**, 108–112.
[73] Garner, F. B., Meyer, C.A., White, D.S. and Lipton, A. (1975) Aerosol BCG treatment of carcinoma metastatic to the lung: A phase I study. *Cancer*, **35**(4), 1088–1094.
[74] Darrah, P.A., Bolton, D.L., Lackner, A.A. *et al*. (2014) Aerosol vaccination with AERAS-402 elicits robust cellular immune responses in the lungs of rhesus macaques but fails to protect against high-dose *Mycobacterium tuberculosis* challenge. *Journal of Immunology*, **193**(4), 1799–1811.
[75] Xing, Z. and Lichty, B.D. (2006) Use of recombinant virus-vectored tuberculosis vaccines for respiratory mucosal immunization. *Tuberculosis (Edinburgh)*, **86**(3–4), 211–217.

Index

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

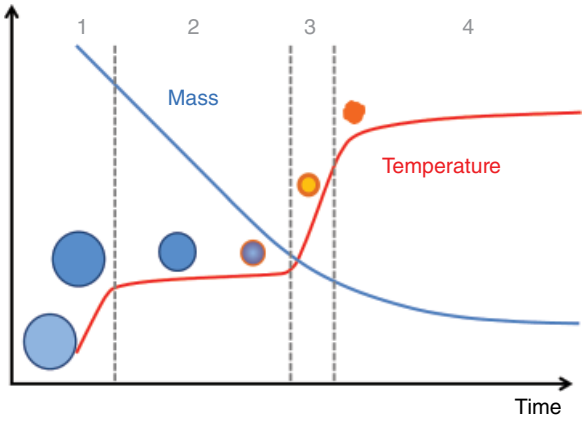


Figure 9.3 *Mass and temperature changes during single-droplet drying inside a spray dryer*

Drug Delivery Systems for Tuberculosis Prevention and Treatment, First Edition.
Edited by Anthony J. Hickey, Amit Misra and P. Bernard Fourie.

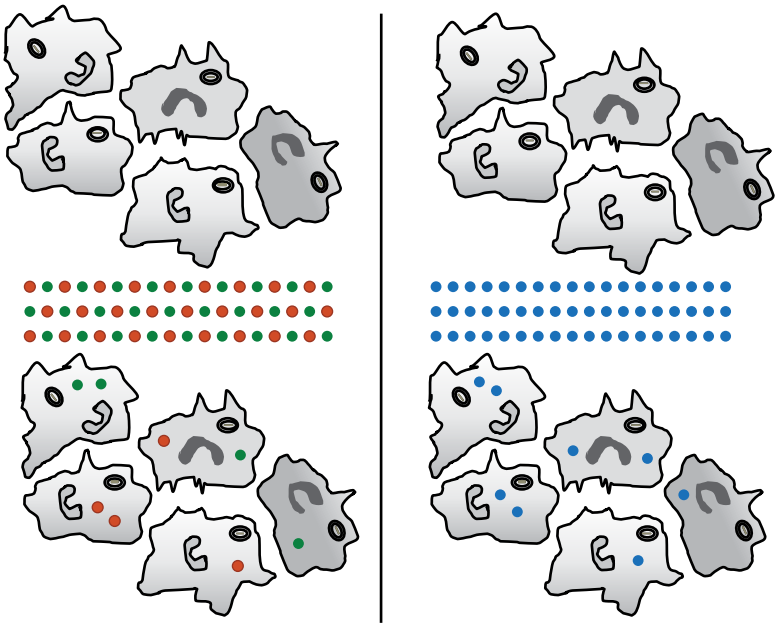


Figure 11.3 *Statistical versus stochastic uptake of particles by infected macrophages*

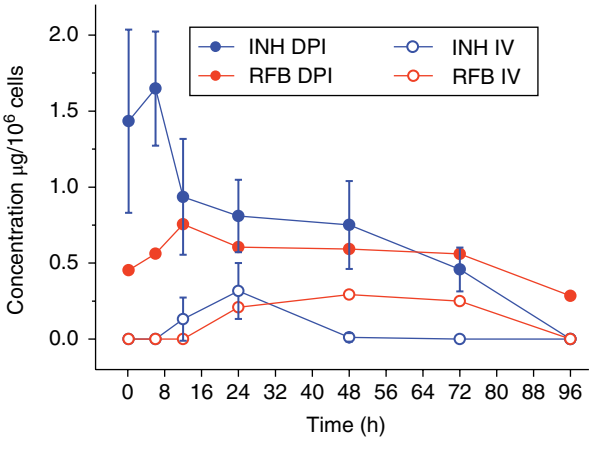


Figure 11.5 *Time course of H (blue) and rifabutin (red) in cells recovered by bronchoalveolar lavage from mice receiving about 100 µg of a dry-powder inhalation (DPI) comprising 1:1:2 parts of H, rifabutin, and PLA or intravenous injections (IV) of the same amounts of drugs*

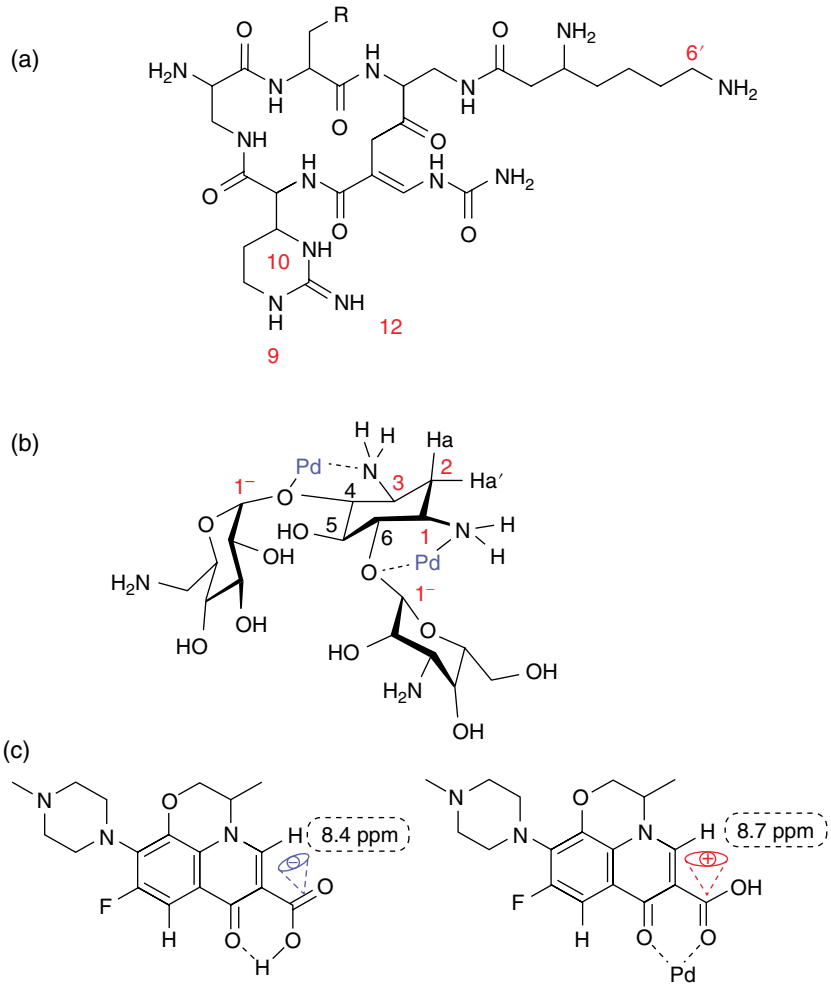


Figure 12.3 *Proposed structures and Pd chelation of (a) capreomycin, (b) kanamycin and (c) ofloxacin obtained by NMR measurements. The nuclei supposedly involved in the interaction with the metal are indicated (adapted with permission from Ref. [72])*

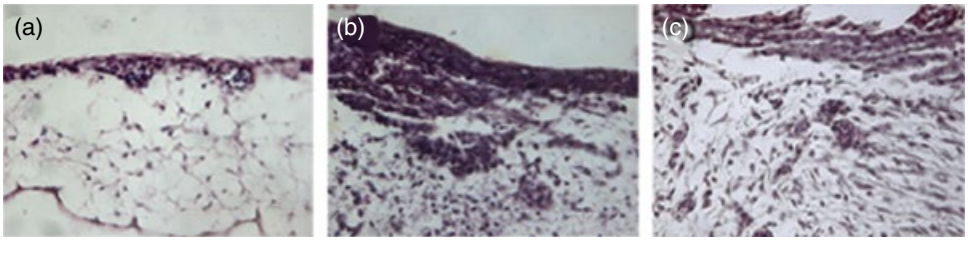


Figure 12.6 *Orthogonal histological sections of chicken embryo chorioallantoic membranes of (a) control group, (b) group treated with capreomycin sulfate and (c) group treated with capreomycin oleate ion pair at day 8. Magnification: 400× (adapted with permission from Ref. [75])*

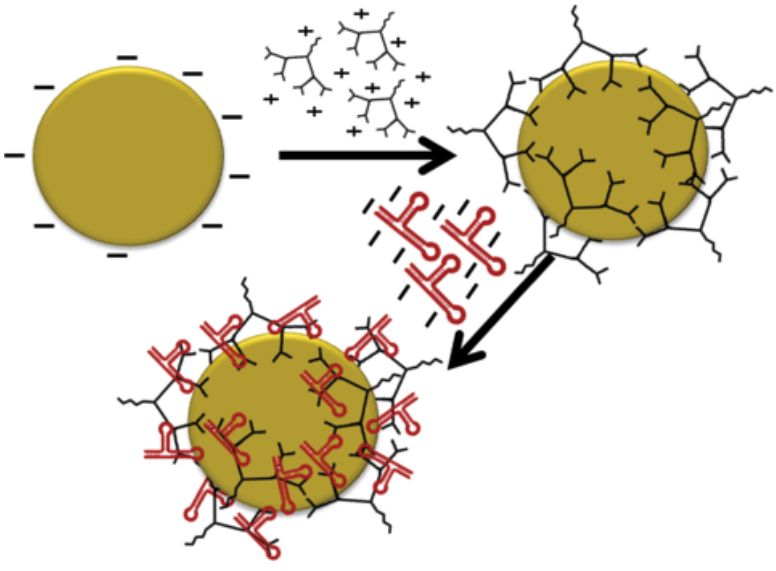


Figure 15.1 *Formation of a nucleic acid (RNA is shown) conjugate where a branched dendrimer structure binds it to the surface of the nanoparticle and protects it from nuclease degradation*

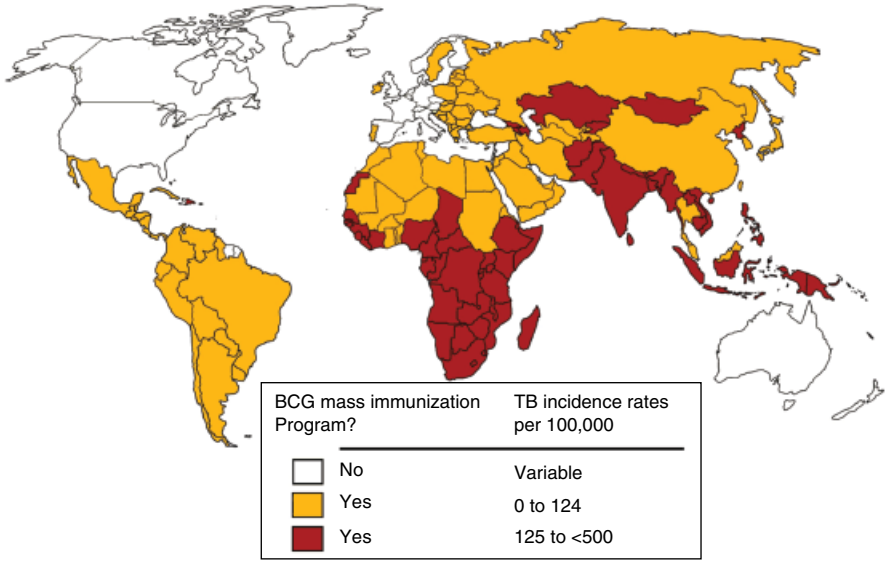


Figure 18.1 *BCG efficacy varies geographically around the world. Colored regions have National BCG Mass Immunization Programs in place, darker colors correspond to the highest TB burden regions*

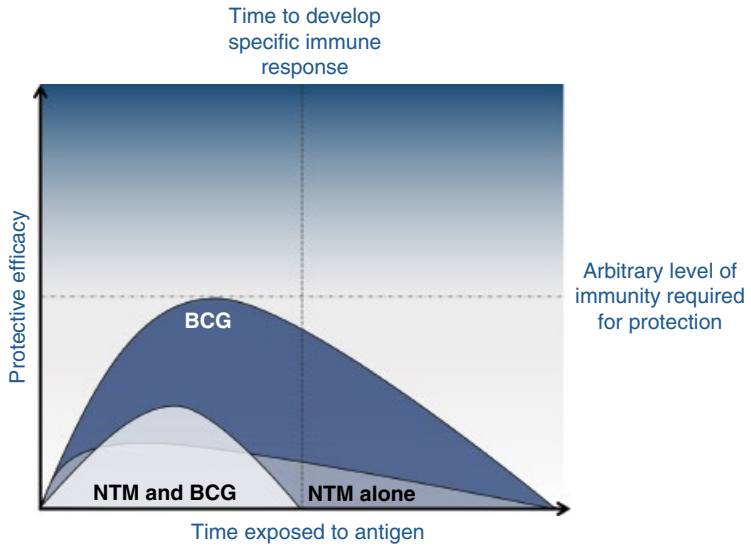


Figure 18.2A Blocking hypothesis*. BCG in naive individuals is able to replicate in the lungs long enough for the development of a BCG-specific immune response to develop. This level of immunity is protective against Mtb challenge (BCG curve). NTMs in naive individuals replicate and generate a NTM-specific immune response; however, this level of immunity is not sufficient for protection against subsequent Mtb infection (NTM alone curve). BCG in NTM-exposed individuals provides insufficient protective immunity against TB. Prior NTM immunity inhibits BCG replication and the generation of a BCG-specific immune response (NTM and BCG curve)*

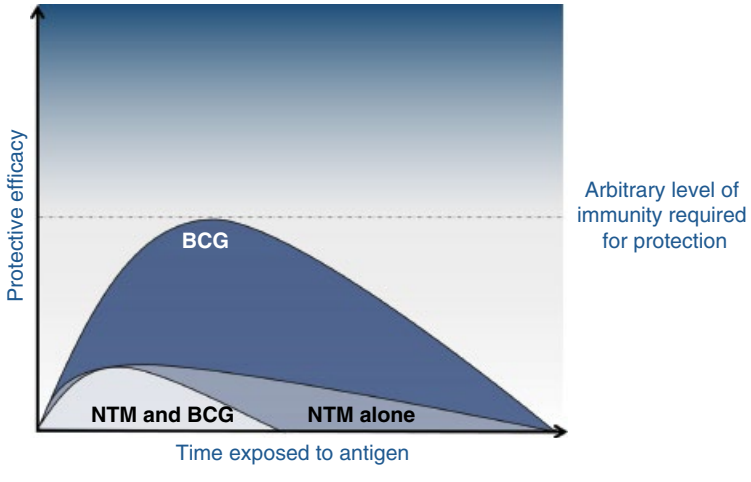


Figure 18.2B Masking hypothesis*. BCG in naive animals provides sufficient protection against Mtb challenge (BCG curve). NTM exposure in naive individuals generates some immunity, but this is insufficient against Mtb challenge (NTM alone curve). BCG in NTM-exposed individuals does not generate immunity above the immunity already generated by NTM exposure, leaving them susceptible to Mtb infection (NTM and BCG curve)*

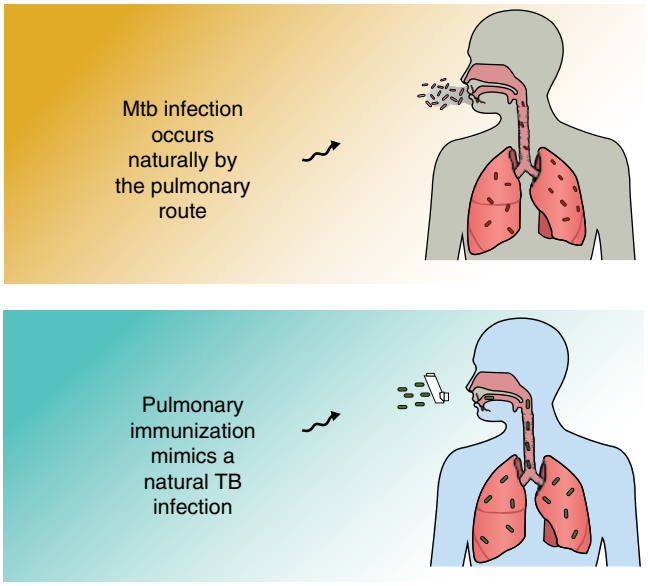


Figure 18.3 ***Biomimicry:*** *Pulmonary immunization with a live bacterial vaccine will mimic a natural infection and provide protection to the host*

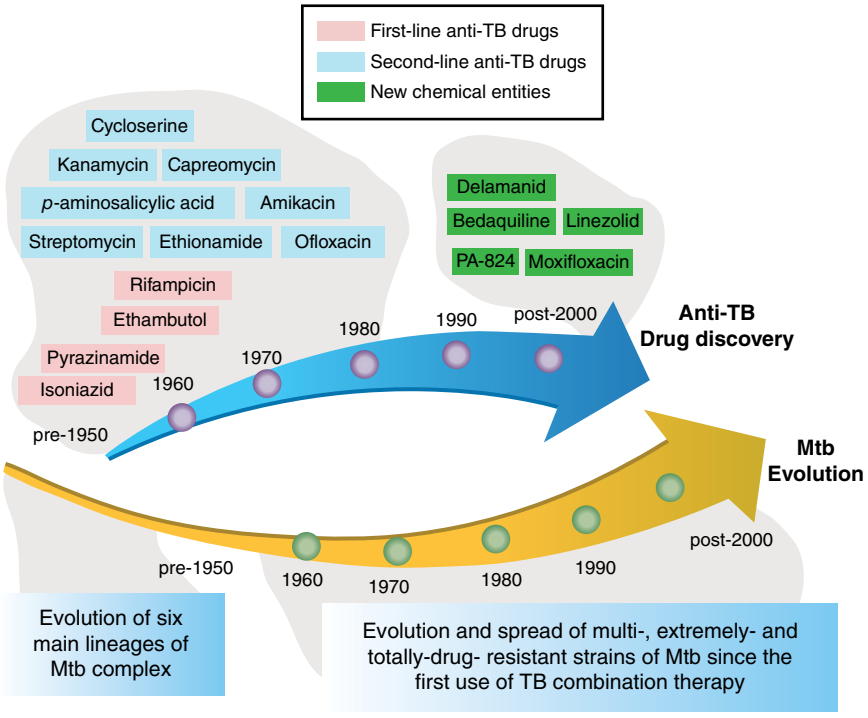


Figure 18.5 *Mtb has the ability to evolve in response to pressure from antibiotics to become drug-resistant. It will continuously adapt and change to the evolutionary pressure and will always be a moving target to new treatment modalities*