Topics in Medicinal Chemistry 11

James R. Empfield
Michael P. Clark *Editors*

Reducing Drug Attrition

Springer

11
Topics in Medicinal Chemistry

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Editors

Reducing Drug Attrition

With contributions by

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Preface

The premise of this book is to provide guidance to those persons who are dedicated to the creation and development of new drugs to aid patients. Our aim was to address the key factors that have led to failure of preclinical and clinical drug candidates. Despite the significant scientific advances over the past few decades in the disciplines associated with research and development (R&D), the overall productivity, as measured by new approved drugs, has not improved. However, the reasons for failure in the clinic have changed over time. Today the two most prevalent factors leading to drug failure are drug safety and lack of efficacy. While clinical failure due to pharmacokinetic factors has been reduced, it is still a significant challenge at times within the drug discovery phase. Although the success rate in delivering important drugs to patients has not yet yielded significant improvements, our understanding of what causes these failures and how to address them has advanced.

This volume does not aim to cover all aspects of drug failure but rather focuses on a few key areas that can address the success rates in pharmaceutical R&D from the selection of the biological target to the safety profiling of the potential clinical candidates. In chapter "Target Selection and Validation in Drug Discovery", Clive G. Jackson tackles the importance of biological target selection and validation including strategies for prioritizing the most appropriate targets for disease intervention. He addresses aspects of the complexity of disease mechanisms, selection of targets in the genomic era, and challenges of clinical trial designs. He also conveys strategies for reducing attrition based on target selection. In chapters "Optimizing Pharmacokinetic Properties and Attaining Candidate Selection" and "The Role of Biotransformation Studies in Reducing Drug Attrition" the authors deal with pharmacokinetic aspects of drug discovery. In chapter "Optimizing Pharmacokinetic Properties and Attaining Candidate Selection", Keith W. Ward focuses on pharmacokinetic related attrition and how to optimize these properties in drug candidates with both in vitro and in vivo studies. In chapter "The Role of Biotransformation Studies in Reducing Drug Attrition", Douglas K. Spracklin et al. address the key issue of biotransformation and its role in drug attrition, including reactive metabolites. Chapter "Reducing Drug Attrition: Safety

Pharmacology" focuses on the key role of safety pharmacology in the identification of potential new drugs. Peter Siegel discusses how to maximize safety pharmacology activities to reduce drug attrition.

It is our hope that the readers will find this book helpful in their drug discovery efforts to fight against diseases.

We would like to thank the authors who have contributed to this treatise for sharing their expertise as well as the time they dedicated to the completion of this volume.

Boston, MA, USA James R. Empfield

Introduction

Attrition: The Biggest Enemy of the Pharmaceutical Industry

The Problem

The research and development (R&D) costs associated with a new drug (NME: new molecular entity) have grown significantly over the past couple of decades. A study undertaken by the US Congressional Budget Office in 2006 found that total spending on health-related R&D had tripled between 1990 and 2006 while NME approvals, following a spike in the mid-1990s had been constant in the range between 20 and 30 [1]. A 2003 analysis by a group at Tuffs University found that the R&D costs associated with new drugs are estimated to be $802 million [2]. However, this is a low estimate when taking into account the total cost (all attrition) in R&D. The cost of developing new drugs has been calculated to be growing at an annual rate of >13% over the past 60 years and increasing at an exponential rate [3]. A major cause of this increase has been the increased failure of drug programs to deliver a marketed drug for therapeutic use [1]. The success rate from potential candidate drug to a marketed product has been estimated to be between 4 and 11% [4]. Considering all attrition, from the start of chemical optimisation in a drug discovery project, the overall success rate is probably closer to 1–2%. The reasons for attrition have changed over time. With the introduction of drug metabolism and pharmacokinetics (DMPK) as primary screens in drug discovery projects since the 1990s, pharmacokinetics (poor bioavailability or metabolism) is no longer the primary reason for failure. Instead, toxicity now accounts for the bulk of pre-clinical project and phase I failures. In the later development phases, lack of efficacy is the primary cause of compound attrition. In addition to toxicity and efficacy, portfolio decision-making is a major contributor to attrition. Within the arena of small molecule drug discovery and development, attrition is highest in the central nervous system (CNS) area and lowest in cardiovascular [5]. Even after market launch, it has been estimated that ~10% of new drugs show unexpected adverse reactions in patients [5]. This book aims to address how drug discovery

organisations can improve success rates (lower attrition) on the road from potential biological target for intervention through the creation of candidate drugs and clinical studies and finally through regulatory approvals to address unmet medical needs. Topics covered in this book are broad, including biological target selection, medicinal chemistry design principles, biotransformation, pharmacokinetic optimisation, safety pharmacology, toxicology, and pharmaceutics.

Advances in Drug Metabolism and Pharmacokinetics (DMPK) and Toxicity Assessment

Over the past couple of decades the drug discovery industry has placed an emphasis on frontloading DMPK and toxicity so as to address these areas prior to clinical development. Within the DMPK area numerous in vitro assays with high throughput have been developed to assess drug candidate permeability, metabolic stability, P-glycoprotein (PgP) active transport, and specific cytochrome P450 inhibition and metabolism. In a similar way, although somewhat more recently, in vitro assays to assess toxicity risks have been established. For example, assessment of cardiac risk of drug candidates is now routinely carried out through various in vitro hERG (human ether-a-go-go gene) evaluations. Similarly, in vitro assessment of genetic toxicity and phosphlipidosis (a lipid storage disorder that leads to excess accumulation of phospholipids in cells) is carried out at many companies. These assays have not only guided the selection of drug candidates for clinical development, but also have enabled a greater understanding of how to design higher quality compounds that are devoid of DMPK and key toxicity liabilities. While this has had a major impact on early drug discovery, particularly opposite DMPK, it has nonetheless only touched the surface of the potential toxicity liabilities that drug candidates may face. Therefore, additional efforts to understand how to design more 'drug-like' agents have been a focus for medicinal chemists over the past decade.

Drug-Likeness, Toxicity and Physical Properties

Recent developments, primarily from studies of large proprietary and public databases, strongly indicate that a number of molecular properties are associated with successful DMPK and toxicity drug discovery outcomes, and control of these properties is indicated as a key activity in the war on attrition. The key findings are briefly summarised here.

The well-known 'rule of 5', derived from a survey of drugs in phase II [6], has stimulated many further studies aimed at linking these easily assessed physical properties with DMPK and more recently toxicity liabilities. The rule of 5 states that absorption of drugs is optimal when: the molecular weight is <500,

lipophilicity (the octanol-water partition coefficient, log*P*) is <5; the sum of oxygen and nitrogen atoms for hydrogen bond acceptors is <10; and the sum of OH and NH groups for hydrogen bond donors is <5. Lower limits were not set in this analysis. This published analysis became a guide for medicinal chemists and drug discovery scientists in their efforts to design drug candidates that would have a greater success rate opposite DMPK properties. Since this report in 1997, other physical properties, namely polar surface area (PSA), rotatable bond count, and ionisation state, have been reported to be relevant parameters for bioavailability [6, 7]. The physical properties of compounds in the various phases of clinical development suggest that there is a convergence towards drug-like properties as compounds proceed towards being marketed [8, 9]. This analysis suggests that both higher molecular weight and more lipophilic compounds tend to be discontinued from development at a higher rate. Despite this finding, a study on the properties of new compounds being patented in current drug discovery projects indicates that they are both larger (higher MS) and more lipophilic than historically approved oral drugs [10]. Amongst oral drugs, there has also been a substantial increase over time in molecular weight and H-bond acceptors, but lipophilicity, H-bond donors and % PSA are not changing significantly [11]. It has been proposed that physical properties that are not changing over time are more important indicators of ultimate success and that lipophilicity is the most critical drug-like property [10, 11].

Recent studies of compounds in differing phases of development show that structural features are also linked to successful outcomes. In the GlaxoSmithKline portfolio, the number of aromatic rings reduces from preclinical to phase III and it was proposed that <3 aromatic rings is preferred; however, the aromatic ring count also correlates with lipophilicity [12]. This is the only report to date showing that attrition in a major pharmaceutical company's portfolio is linked to compound lipophilicity. The 3-dimensionality of molecules also appears to be important; a simple measure, the fraction of tetrahedral carbon atoms (sp^3 hybridised carbon atoms), increases through the development phases [13]. A similar trend was found with structural similarity to natural products and metabolites; a 'biological relevance' parameter increases though the phases of drug development [14].

The importance of optimal drug-like properties has been reinforced by studies relating physicochemical properties to empirical DMPK and toxicity [15] parameters. Amongst AstraZeneca and Pfizer proprietary compounds, both molecular weight (Mol Wt) and lipophilicity (log*D* at pH 7.4) influence compound permeability in CACO-2 cells [16, 17]. As Mol Wt increases, higher log*D* is needed to maintain a better than even chance of reaching high permeability [16]. Considering permeability and metabolic stability together, optimal properties were found at Mol Wt 350 and log*D* 1.5 [17], values very similar to mean oral drugs [8]; increasing Mol Wt to >450, however, results in a marked reduction in combined permeability and stability [17]. In a study of GlaxoSmithKline proprietary compounds, focussing on solubility, permeability, bioavailability, volume of distribution, clearance, PGP efflux, cytochrome P450 inhibition and hERG inhibition, it was found that compounds with molecular weight <400 and log*P* <4 generally had improved risk profiles compared to those with greater values [18]. A study of AstraZeneca

compounds shows that hERG (human ether-a-go-go gene) inhibition, an indicator for cardiovascular toxicity, is primarily driven by compound's lipophilicity and basicity [19]. In 245 compounds assessed for in vivo toxicity in Pfizer, overall organ toxicity, when normalised for exposure, was shown to be reduced by 6-fold when clog*P* is <3 and PSA is >75 [20]; the effect is larger (24-fold) for bases, perhaps due to higher volumes of distribution. A study by AstraZeneca of the Bioprint database, where 2,133 drugs and reference compounds have been examined in >200 assays, shows that promiscuity is linked to clog*P*: values of <3 demonstrate reduce risk while those >4 have increased risk [10]. Basic compounds showed a much higher risk of promiscuity (polypharmacology) than neutral or acidic compounds, but in all classes, promiscuity increased with lipophilicity. The metric ligand lipophilicity efficiency (LLE = p(Activity) – log*P* or *D*) has been proposed as a simple measure to assess the quality of drug candidates; values of LLE of >5 are expected to demonstrate lower risks of promiscuous behaviour and therefore lower toxicity [10]. Similar trends in promiscuity were seen among a set of Roche compounds [21] where promiscuity of bases was ascribed to the presence in the screening panel of amine-preferring targets, for example aminergic G-protein coupled receptors.

Overall, the studies summarised above should help to guide medicinal chemists in their efforts to design higher quality compounds and avoid the more obvious issues linked to compound failures. In essence, many studies now confirm that physical properties decrease in the order: research compounds > development compounds > drugs. To maximise the success of drug discovery programmes design work should be carried out in the optimal regions, rather than the far-flung zones, of drug-like space, especially in consideration of lipophilicity. It is clear that combining high lipophilicity with basicity is likely to markedly increase the risk of toxicity and promiscuity. This necessitates that lead generation activities should be focused to seek the right properties to begin chemical optimisation. It has been suggested that high throughput screening (HTS), with a focus on finding active compounds, has led to the selection of larger and more lipophilic, and therefore less viable, leads [22]. Assessment of HTS hits by their ligand efficiencies, which is essentially potency corrected for physical properties, is an essential step in improving lead-like [23] quality. The field of fragment-based drug discovery, which relies on highly ligand efficient but small fragments as starting points, has made considerable progress [24]. In principle, starting optimisation with a fragment should make it easier to stay closer to the optimal regions of drug-like space. Drug targets being pursued today are different from historical targets and while target class differences in physical properties do exist [25], these differences are no greater than the differences seen between the patent profiles of individual companies [10]. In addition, the spread of log*P* values in each target class is greater than the differences between target classes. These observations suggest it should be possible to find compounds with optimal physical properties to reduce DMPK and toxicity risk with the bulk of drug discovery targets.

Target Selection and Validation

The genomic revolution has provided drug discoverers with knowledge of the full complement of human genes and proteins. However, this is not the panacea that it may seem. Firstly, only a small proportion (~3,000) of the genome is likely to be susceptible ('druggable') to small molecule drug discovery [26]. Secondly, linking individual target proteins to disease states (validation) remains a formidable challenge and selecting the wrong target for a disease will inevitably result in attrition due to efficacy. To overcome this, pathway-based discovery has been proposed as an alternative approach [27]. Manipulating pathways could have implications for therapy beyond targeting single diseases [28]. Recently there has been a surge of interest in developing drug-target relationships or networks [29, 30] which have demonstrated that drugs are rarely completely selective for a single target. Antipsychotic drugs, which rely for their effectiveness on binding to several monoaminergic G-protein coupled receptors [31], provide a classical example of polypharmacology, albeit serendipitous in that these profiles were unknown for the most part when these drugs were discovered. Structure-based polypharmacology, *deliberately* targeting multiple proteins, may offer a means of lowering attrition due to efficacy [32]. In following this path, care will have to be taken to avoid introducing physical chemistry driven promiscuity with accompanying risk of toxicological failure. Optimising desired multiple activities in single molecules remains an important future design challenge [33] for medicinal chemists.

Prospects

The very high attrition rates in pharmaceutical pipelines, while being a major issue for the health of drug R&D, also offer an opportunity: *a 10% improvement in success rate would more than double the output of new medicines*. There are reasons to be optimistic that this can be achieved.

Significant progress has been made within the drug discovery industry in addressing attrition due to DMPK properties and specific toxicity liabilities, such as drug interaction with the hERG cardiac channel. It is clear that the developments in small molecule drug discovery, outlined above, are beginning to provide better understanding of the root causes of failure resulting from inherent molecular properties in candidate drug molecules. In particular, minimising the physical property risks associated with toxicity and drug metabolism has the potential to impact attrition rates substantially in the pre-clinical phase. Molecular properties are entirely under the control of medicinal chemists and are determined by the lead selection strategies and optimisation tactics chosen by drug discovery projects.

Ensuring appropriate drug exposure for binding to the target receptor and sufficiently wide safety margins to allow dose escalation, combined with measurement of relevant clinical biochemical responses, can begin to address the failures

seen in early clinical phases. Improved understanding of target pathways and linkage to disease states will improve efficacy failure and potential mechanism-based toxicity (toxicity due to interaction of the biological target of interest). Addressing both toxicity and efficacy liabilities has the promise of decreasing drug attrition over the next decade and beyond.

References

1. Austin DH (2006) A congressional budget office study: research and development in the pharmaceutical industry. www.cbo.gov
2. DiMasi JA, Hansen RW, Grabowski HG (2003) The price of innovation: new estimates of drug development costs. J Health Econ 22(2):151–185
3. Munos B (2009) Lessons from 60 years of pharmaceutical innovation. Nat Rev Drug Discov 8 (12):959–968
4. Kola I, Landis J (2004) Opinion: can the pharmaceutical industry reduce attrition rates? Nat Rev Drug Discov 3(8):711–716
5. Schuster D, Laggner C, Langer T (2005) Why drugs fail – a study on side effects in new chemical entities. Curr Pharm Des 11(27):3545–3559
6. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 23(1–3):3-25
7. Schuster D, Laggner C, Langer T (2008) Antitargets. In: Vaz RJ, Klabunde T (eds) Methods and principles in medicinal chemistry. New York:Wiley-VCH, Chap 1, p 3
8. Veber DF, Johnson SR, Cheng H-Y, Smith BR, Ward KW, Kopple KD (2002) Molecular properties that influence the oral bioavailability of drug candidates. J Med Chem 45 (12):2615–2623
9. Martin YC (2005) A bioavailability score. J Med Chem 48(9):3164–3170
10. Wenlock MC, Austin RP, Barton P, Davis AM, Leeson PD (2003) A comparison of physiochemical property profiles of development and marketed oral drugs. J Med Chem 46(7):1250–1256
11. Tyrchan C, Blomberg N, Engkvist O, Kogej T, Muresan S (2009) Physicochemical property profiles of marketed drugs, clinical candidates and bioactive compounds. Bioorg Med Chem Lett 19(24):6943–6947
12. Leeson PD (2007) Springthorpe, Brian. The influence of drug-like concepts on decision-making in medicinal chemistry. Nat Rev Drug Dis 6(11):881–890
13. Leeson PD, Davis AM (2004) Time-related differences in the physical property profiles of oral drugs. J Med Chem 47(25):6338–6348
14. Ritchie TJ, MacDonald SJF (2009) The impact of aromatic ring count on compound developability – are too many aromatic rings a liability in drug design? Drug Discov Today 14(21–22):1011–1020
15. Lovering F, Bikker J, Humblet C (2009) Escape from flatland: increasing saturation as an approach to improving clinical success. J Med Chem 52(21):6752–6756
16. Kong D-X, Ren W, Lu W, Zhang H-Y (2009) Do biologically relevant compounds have more chance to be drugs? J Chem Inf Model 49(10):2376–2381
17. Price DA, Blagg J, Jones L, Greene N, Wager T (2009) Physicochemical drug properties associated with in vivo toxicological outcomes: a review. Expert Opin Drug Metab Toxicol 5 (8):921–931
18. Waring MJ (2009) Defining optimum lipophilicity and molecular weight ranges for drug candidates-molecular weight dependent lower logD limits based on permeability. Bioorganic & Medicinal Chemistry Letters 19(10):2844–2851

19. Johnson TW, Dress KR, Edwards M (2009) Using the Golden Triangle to optimize clearance and oral absorption. Bioorg Med Chem Lett 19(19):5560–5564
20. Gleeson MP (2008) Generation of a set of simple, interpretable ADMET rules of thumb. J Med Chem 51(4):817–834
21. Waring MJ, Johnstone C (2007) A quantitative assessment of hERG liability as a function of lipophilicity. Bioorg Med Chem Lett 17(6):1759–1764
22. Hughes JD, Blagg J, Price DA, Bailey S, DeCrescenzo GA, Devraj RV, Ellsworth E, Fobian YM, Gibbs ME, Gilles RW, Greene N, Huang E, Krieger-Burke T, Loesel J, Wager T, Whiteley L, Zhang Y (2008) Physiochemical drug properties associated with in vivo toxicological outcomes. Bioorg Med Chem Lett 18(17):4872–4875; MacKenzie R (2008) Presented at the 9th annual drug discovery leaders summit, Montreux
23. Peters J-U, Schnider P, Mattei P, Kansy M (2009) Pharmacological promiscuity: dependence on compound properties and target specificity in a set of recent roche compounds. ChemMedChem 4(4):680–686
24. Keseru GM, Makara GM (2009) The influence of lead discovery strategies on the properties of drug candidates. Nat Rev Drug Discov 8(3):203–212
25. Teague SJ, Davis AM, Leeson PD, Oprea T (1999) The design of leadlike combinatorial libraries. Angew Chem Int Ed 38(24):3743–3748
26. Murray CW, Rees DC (2009) The rise of fragment-based drug discovery. Nat Chem 1(3):187–192
27. Vieth M, Sutherland JJ (2006) Dependence of molecular properties on proteomic family for marketed oral drugs. J Med Chem 49(12):3451–3453
28. Al-Lazikani B, Gaulton A, Paolini G, Lanfear J, Overington J, Hopkins A (2007) The molecular basis of predicting druggability. Chem Biol 3:804–823
29. Hellerstein MK (2008) A critique of the molecular target-based drug discovery paradigm based on principles of metabolic control: Advantages of pathway-based discovery. Metab Eng 10(1):1–9
30. Hellerstein MK (2008) Exploiting complexity and the robustness of network architecture for drug discovery. J Pharmacol Exp Ther 25(1):1–9
31. Paolini GV, Shapland RHB, van Hoorn WP, Mason JS, Hopkins AL (2006) Global mapping of pharmacological space. Nat Biotechnol 24(7):805–815
32. Keiser MJ, Setola V, Irwin JJ, Laggner C, Abbas AI, Hufeisen SJ, Jensen NH, Kuijer MB, Matos RC, Tran TB, Whaley R, Glennon RA, Hert J, Thomas KLH, Edwards DD, Shoichet BK, Roth BL (2009) Predicting new molecular targets for known drugs. Nature (Lond, UK) 462(7270):175–181
33. Roth BL, Sheffler DJ, Kroeze WK (2004) Magic shotguns versus magic bullets: selectively non-selective drugs for mood disorders and schizophrenia. Nat Rev Drug Discov 3(4):353–359

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Target Selection and Validation in Drug Discovery

Clive G. Jackson

Abstract The failure of drug discovery productivity to match increased investment chiefly for reasons of poor efficacy in the clinic, despite elucidation of the human genome and growing knowledge of disease processes, has been a cause of much concern to pharmaceutical companies, investors and regulators. The reasons for this poor productivity are wide ranging and include strategic, commercial, regulatory as well as scientific factors. This chapter examines likely root causes and the vigorous response in many areas of drug discovery. These include improved target identification and validation technologies, network pharmacology, greater collaborative ways of working and use of biomarkers and patient stratification. Other areas of potential importance are highlighted such as product positioning and support beyond Phase 3. More than 10 years of experience in the pharmaceutical industry in target selection and validation is drawn on to identify factors important for reducing attrition in the early pipeline including organisation, multidisciplinary input and target selection criteria. Overall there are reasons to be hopeful that these recent developments in target validation and drug development will deliver improvements in productivity that will be sufficient to begin to restore confidence in the sector.

Keywords Target identification, Target selection, Target validation

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1 Introduction

The start of the new millennium was accompanied by optimism that there would be a surge in new medicines to meet patient needs driven by our knowledge of the full complement of human genes and ability to measure their expression in disease. However the next 10 years revealed a very different picture. Investment has

increased but the rate of delivery of new medicines to the market has remained constant, and as a result drug discovery is now less cost-efficient than before the genomic revolution. Whilst the number of projects entering Phases 2 and 3 has increased, the percentage failure rate has increased also. The fact that this higher attrition is almost totally due to poor efficacy has made improving the process of selecting and progressing the right target to meet patient need a critically important issue. Moreover the focus in the industry on reducing drug discovery cycle time and increasing the "number of shots on goal" to improve output has generated an increased demand for targets. In recent years this flat line delivery of new medicines despite increased investment has contributed to reduced investor confidence in the sector and driven major change in the larger pharmaceutical companies including mergers and reduction in their internal research capacity.

This chapter seeks to explore the root causes of this high attrition in Phase 2 and 3 due to poor efficacy. These potential causes are wide ranging and likely to vary between targets, disease indications and pharmaceutical companies. Whilst many are scientific in nature, others are the result of the commercial environment in which companies operate and drug discovery productivity is undoubtedly influenced by a complex interplay of these factors. Ways in which drug discovery research is making a vigorous response on a number of fronts to this challenge are also discussed. That the industry can respond effectively to bring attrition rates down has been demonstrated in the past by the efforts put into front loading preclinical studies of pharmacokinetics and bioavailability that reduced attrition in Phase I due to these factors from 40% in 1990 to 10% today.

Some important concepts underpinning the discussion here are, firstly, that it is not the target that is being validated but the hypothesis of how modulating target activity can deliver efficacy. A target may be invalid in one hypothesis but valid in another, e.g. the differing effects of anti-TNF therapy in multiple sclerosis compared to rheumatoid arthritis. Secondly, as it is not possible for target validation strategies to deliver absolute confidence ahead of Phase 2 and 3 clinical trials, the objective is ultimately one of risk management where a suitable balance needs to be struck between the cost and time taken and the amount of risk reduction achieved. Thirdly, a therapeutic target is often thought of as a single protein that is directly involved in a causative disease process. However it is important to think more widely than this. A target need not be part of a disease mechanism but can be part of a normal physiological process that is able to suppress symptoms if harnessed through drug intervention, e.g. β2-adrenoreceptor agonists are used as bronchodilators in asthma. Today target scope has increased beyond proteins to include messenger RNAs and the growing number of classes of RNA regulatory elements such as microRNAs by employing oligonucleotide therapeutics. Also increasingly it is necessary to consider how targets in combination may be needed to deliver the desired benefit to patients.

Ten years of experience of selecting and further validating targets in respiratory and inflammatory diseases for small molecule, monoclonal antibody and oligonucleotide drug discovery at AstraZeneca will be drawn on to identify some success factors in lowering early drug discovery pipeline attrition. The importance will

be highlighted of a clear definition of the hypothesis describing the molecular and cellular mechanisms linking the target to the clinical trial end points and how multiple sources of evidence can be integrated to increase or decrease confidence in this hypothesis. Also evidence is presented of the positive impact on drug discovery pipeline performance of increasing the stringency of target selection and validation criteria and of operating in multidisciplinary teams dedicated to target delivery against a clearly defined target product profile.

Before starting it is necessary to address the matter of terminology. Target identification, target selection, target validation and translational research are just some of the terms used widely and differently in the literature. For the purpose of this chapter, target identification refers to the process by which a candidate gene becomes prioritised for further evaluation, whilst target selection refers to the point at which sufficient evidence has been gathered for the target to be accepted into the drug discovery pipeline and initiate hit identification. Target validation will be used to describe the process that continues right from target identification up to launch (and beyond) of gathering supporting evidence that therapeutic intervention will deliver the expected efficacy in patients. Translational research in the context of drug discovery is a rather broad term that has come into use to cover a range of strategies to more effectively integrate preclinical and clinical research to achieve rapid and successful translation of new research discoveries about disease into treatments for patients.

2 Extent of the Challenge

The stark facts of Pharmaceutical R&D productivity are that the introduction of new molecular entities (NMEs) has remained roughly constant for the last decade despite a major rise in R&D investment by large pharma which exceeded $50 billion dollars in 2010 [1]. It has been estimated to cost on average $1.2–1.3 billion dollars to bring an NME to the market when the cost of failed projects is included [2]. The overall failure rate in pharmaceutical development for compounds from first time in man to registration has remained at around the 90% level or higher over the last decade. The top ten pharmaceutical companies have on average produced less than one registered NME per company each year between 2005 and 2010 [3]. This shows an overall decline in Pharmaceutical R&D productivity. The number of clinical trials has increased especially at Phase 2 and 3, which are the most expensive development stages, and this is a major factor in driving up R&D spending. Between 1990 and 2004 attrition increased from 40 to 70% at Phase 2 and from 20 % to 55% in Phase 3 [3]. At the heart of this problem is failure due to poor efficacy which in recent years accounted for 50% of all Phase 2 and Phase 3 failures and was the dominant factor driving attrition. Of the remainder safety was the next largest cause which may also include a component due to target-related adverse effects which is also a target selection and validation issue.

This stagnation in the number of new drug launches seems particularly surprising given the advances in disease research, the publication of the human genome and the rapid growth in the biopharmaceutical sector. However it is important to recognise that today new medicines face higher regulatory and safety assessment hurdles than in the past. Therefore it can be argued that drug discovery has maintained a constant output whilst improving quality. Meeting these standards has inevitably generated upward pressure on development costs despite drives for increased efficiency and reduced cycle time in the industry.

The initial finding that monoclonal antibodies delivered up to threefold higher success rate in development than small molecules [4] has driven major investment in this area and an increase in number of projects entering the clinic. However it remains to be seen if this improvement can be sustained long term as drug developers move beyond the few early high confidence targets into less well-validated ones in order to fuel the expansion of the biopharmaceutical sector. Also the high pricing of biologicals is a further threat to their continued success given the increasing pressure on private and public payers to control costs. Therefore biologicals will need to be aimed at indications with high unmet need and show very good cost-effectiveness in order to be viewed positively by them.

Currently only around three in ten launched products recover their development costs [5], and with pricing likely to become an ever more keenly contested issue by payers around the world, this could result in an increase in the effective failure rate. The situation is exacerbated by the fact that several pharmaceutical companies are facing an imminent loss of patent cover on many of their most profitable medicines. Therefore, despite the current profitability of large pharmaceutical companies, the sector is causing concern to investors. In an attempt to respond to these challenges, management in the sector has turned to a variety of measures including mergers and consolidation, reduction of the cost base of research and development, externalisation of research and collaborations and public private partnerships to drive innovation and improve future productivity.

3 Root Causes

Analysis of why so many projects fail due to lack of efficacy in Phases 2 and 3 is important if we are to avoid repetition in the future, but it can be hard to establish exact reasons, and failure can be due to a combination of factors. However there is evidence to suggest that the following are some frequent contributory causes for failure. These include overdependence on single technologies or paradigms for target selection, lack of understanding of disease processes and their underlying molecular and cellular complexity, lack of predictive biological test systems in vitro and in vivo and lack of tools to effectively probe gene function. It is also likely that aspects of clinical trial design including inability to predict responder patient subsets have played a key role. Strategic management decisions and commercial pressures in the sector have themselves contributed to the observed

attrition. At the same time there has been a growing requirement for higher safety standards than was the case 10 years ago.

3.1 Increased Risk in Drug Discovery Portfolios

A recent careful study of the 28,000 drug development compounds in the Pharmaceutical Industry Database of the IMT Institute for Advanced Studies, Lucca, Italy, points to a move away from drug discovery in therapeutic indications with a higher probability of success, e.g. cardiovascular disease and acute, non-life-threatening indications into higher-risk areas such as CNS disorders, oncology and chronic diseases such as COPD. Quantitative assessments of this effect indicated that this alone could explain the reduction in pharmaceutical productivity [3]. Important driving forces for this trend are the greater potential financial returns and lower competition. Indications with high probability of success tend to be those with existing drug launches and a well-established development path in the clinic. Payers are willing to reward novel, differentiated therapies for areas of unmet need with higher pricing. However, best-in-class products showing incremental improvements over existing products, even though of value to the patient, may be subject to reference pricing schemes and competition from generics. Also the reduced risk of failure of best-in-class medicines has to be set against the cost and risk of larger Phase 3 studies needed by some regulatory authorities to demonstrate advantage over existing therapies and the ability of first-in-class products to maintain their market share against competition from second- and third-in-class launches [6]. At the same time, the growth of genomic technologies and information about disease pathways has given the industry a large number of potential first-in-class target opportunities in these areas of unmet need. Given that this reimbursement picture is unlikely to change the sector needs to be successful in first-in-class and higher-risk areas both for its future profitability and to benefit patients who are as yet without effective treatments.

There can be strong commercial pressure for pharmaceutical companies to show analysts and investors a full portfolio of candidate drugs in development. Also large pharmaceutical organisations aim to maintain a full portfolio of candidate drugs in the clinic to fully utilise their preclinical research and development capacity. Therefore strategic decisions are sometimes made to take high-risk projects through Phases 2 and 3. Such decisions may also be taken due to the perceived high financial benefit of succeeding in a new area of unmet need. A further reason for project attrition due to strategic decision-making can result from changes in disease area focus. In some cases this has caused closure of projects as late as Phase 3 and registration. Given the long cycle times in drug discovery, frequent changes in disease area focus can incur considerable loss of efficiency.

3.2 Payer Willingness to Pay

A positive Phase 3 study is often seen as the successful conclusion to the drug discovery process. However a considerable hurdle still remains even for a differentiated, first-in-class medicine which is the willingness of health-care providers to pay and advisory bodies such as the National Institute for Clinical Excellence in England and Wales to recommend the treatment.

An example of attrition due to failure to convince the payers and advisory bodies of the cost-effectiveness of a medicine is Pfizer's product Exubera. Here there was an expectation that inhaled insulin would improve quality of life through greater convenience, also encourage poorly controlled diabetics on oral therapies to include insulin in their treatment regime and would lead to improved metabolic control. Although Exubera was as effective in glycaemic control as subcutaneous insulin and was preferred to traditional injections, no clinical advantage was shown, and costs were much higher due to poorer bioavailability. Payers declined to take up the product due to a perceived lack of cost-effectiveness [7]. Further long-term trials to demonstrate improved metabolic control through better compliance may have been able to provide important further support if undertaken. The increased convenience of insulin pen injectors was likely to further erode any advantage of Exubera as these were actually preferred in a later study [8]. This example shows how important it is to have a clear well-researched product vision (usually described as a target product profile, TPP) that correctly identifies unmet patient need, aligns with strong drivers for cost-effectiveness and takes into account potential competitor developments.

3.3 Complexity of Disease Mechanisms

Major progress is being made in identifying many molecular and cellular changes that occur in disease. However our understanding is clearly incomplete in most diseases where there is unmet need as shown by the high failure rate at Phase 2 for first-in-class treatments. The heterogeneity of patient responses to new medicines further underlines the need to understand how disease mechanisms vary between patient segments and longitudinally with time as the disease progresses.

Cancer might have been expected to be an early area of success as comparison of gene sequences between health and disease has led to the identification of many oncogenic and tumour suppressor mutations that can be shown to be present in certain patient subsets [9]. The development of a number of targeted therapies against certain oncogenes has followed, but in the view of many oncologists, the impact has been less than expected [10]. There have been notable successes such as in the treatment of chronic myelogenous leukaemia with Gleevec [11], but most cancers develop resistance to these treatments that limits their value, e.g. to the EGFR inhibitors Tarceva and Iressa in non-small cell lung cancer [10].

The recognition of the importance of neovascularisation in solid tumour development has generated much effort in antiangiogenic approaches. However the anti-VEGF monoclonal antibody Avastin has been approved for use in certain solid tumours such as colorectal cancer but requires combination with chemotherapeutic agents. Results with other tumours have been negative or marginal, and the FDA recently withdrew approval for Avastin use in breast cancer which underlines the mechanistic complexity and differences between solid tumours [12].

Hypotheses about pivotal disease mechanisms dictate the biological test systems used to make target selection and validation decisions. For a long time the increased proliferation of cancer cell lines was considered as a cardinal feature of the disease and was used to test for effective treatments. However the more recent observations that primary tumour cells frequently have slow proliferation rates but show increased resistance to programme cell death and are often more sensitive to compounds that induce apoptosis than normal cells [13, 14] have shifted the screening paradigm. This emphasis on rapid growth may have contributed to the poor performance of many earlier agents in the clinic.

Now there is strong investment in personalised medicine approaches in many diseases [15, 16]. Also there is an important movement to accelerate this growth of disease understanding further by more open and collaborative working (see Sect. 6). Technological advances in genomic sequencing, informatics, genetics, epigenetics, imaging and RNAi, amongst others, are also making their contribution to advancing disease understanding at the present time (described in Sect. 9).

3.4 The Dominance of a Single Paradigm or Technology

3.4.1 A Historic Perspective

It is important to recognise how the drug discovery process and target selection strategies have been strongly influenced by the latest advances in biological research over the last 30 years.

Before 1980 drug discovery was largely based on the screening of compounds for a desired response in cells or in an animal efficacy model (currently known as a phenotype-led approach). Subsequently the target became characterised by painstaking biochemical studies. A good example is FPL 55712 that was discovered as an antagonist of the then uncharacterised slow-reacting substance of anaphylaxis and contributed to the characterisation of the type 1 cysteinyl leukotriene receptor (cysLT1) and its ligands. Medicinal chemistry approaches lead ultimately to the development of Accolate and Singulair as important treatments for asthma [17].

Subsequently drug discovery shifted to focus on identifying targets directly through a growing, but still limited, understanding of signalling pathways, receptors and ligands playing a role in disease such as the enzymes of the arachidonic acid cascade. Subsequently the race to clone new examples of key druggable target classes such as GPCRs and kinases by homology led to the “target class approach”

where attempts were made to secure patent protection and find applications in disease for new family members [18]. The opportunity to grow expertise in the biology and methods of a given target class and develop directed libraries of small molecules were seen as major advantages. This approach has given rise to important areas of drug research such as the molecular characterisation of the many serotonin receptor subtypes, the elucidation of their roles in the central and peripheral nervous system and medicines for migraine and depression [19]. However it could also fuel research spending not directed against any specific patient need, and there could be wasted effort in trying to find suitable indications for new members of the class.

3.4.2 Target Selection in the Genomic Era

The release of the first draft of the human genome [20] in conjunction with the availability of powerful tools such as gene array chips (described further in Sect. 9.2) for comparison of mRNA expression levels for all genes in the genome in cells and tissues in health and disease was expected to bring about a revolution in understanding of disease processes and discovery of new effective treatments. In turn this fuelled major research investments by pharmaceutical companies in genomic target identification internally and through expensive collaborations [21], e.g. the $465 M Bayer and Millennium collaboration.

However subsequent experience resulted in a major lowering of expectations. There were a number of reasons for the failure of large genomic campaigns to deliver the expected products. Firstly, there was the problem that many hundreds of genes can be uniquely expressed or up- or downregulated in a comparison study between health and disease, and functional analysis of genes was severely rate limiting. This led, in some cases, to large numbers entering drug discovery screening and undergoing later attrition (450 new targets in the Bayer-Millennium study gave rise to 180 targets entering drug discovery and 2 clinical candidates) [21]. Alternatively, potential targets required extensive further filtering involving comparisons of multiple genomic expression datasets from different sources, data mining and application of criteria such extent of upregulation which could result in the loss of valuable targets that, for example, may be regulated mainly by post-translational processing. From experience at AstraZeneca Charnwood in asthma and rheumatoid arthritis, and of others elsewhere, this approach often led to large strategic investments in expression profiling taking around 2 years to generate an output that then proved difficult to progress for various reasons. Retrospective analyses of this period have tended to conclude that this was not a cost-effective approach. However the first targets that can be considered to be of truly genomic origin did enter the clinic [21].

Now genomic expression data is tending to make a more appropriate contribution to target selection, validation and safety assessment through integration with information from other technologies to generate an overall platform of evidence

supporting the hypothesis for how modulating the target will deliver efficacy (see Sect. 9).

3.4.3 The "One Target, One Disease" Paradigm

An underlying tenant of drug discovery that has become prevalent in industry and academia, perhaps inadvertently due to the genomic era, is that for each disease or TPP there will always be single targets that if modulated by a highly selective drug will give the desired efficacy. However this has become increasingly questioned over the last 10 years, and there is much evidence to support the view that combinations of drugs or drugs targeting the activities of multiple genes (called polypharmacy) are likely to be a more productive approach in some if not most situations [22].

At a theoretical level, modelling of biological networks controlling cell phenotypes predicts that these systems are robust due to redundancy, and compensating mechanisms and targeting multiple components in a system is frequently necessary to achieve a change in response of the system [22]. There is preclinical and clinical evidence that this is the case. Lack of phenotypes for the large majority of single gene knockouts in mice and yeast shows the ability of biological systems to compensate for loss of a single component, whilst loss of a further gene can sometimes result in display of a phenotype illustrating the degree of robustness of biological systems [23]. A further elegant demonstration of the importance of the interaction of target combinations is the discovery of synthetic lethal targets in oncology. In this one target in the cell system is mutated, and modulating the activity of the synthetic lethal target can kill cancer cells harbouring the oncogenic mutation whilst having no effect on the untransformed isogenic cell without the mutation although much of the transcriptome is the same in both [24, 25].

Clearly there are examples of good efficacy by selective therapies to a single target, e.g. anti-TNFs. However, in the clinic the importance of combination therapy has been recognised for a long time in many diseases such as asthma and cancer, and there is evidence that many licensed drugs act at multiple targets. It is a striking fact that many of our most effective antibiotics would not have been discovered if the approach had been taken of developing selective compounds against a single target; e.g. fluoroquinolone antibiotics need to inhibit both ParC and GyrA [22]. An interesting demonstration of the importance of the combination paradigm over the actual nature of the targets themselves comes from the BeSt trial in early RA where two different combinations (methotrexate and prednisone or methotrexate and the anti-TNF antibody infliximab) were equally effective at controlling symptoms and preventing joint damage, and both combinations were more effective than optimisation of monotherapies including infliximab [26].

It is hard to estimate what the consequences of the emphasis on this paradigm have been. Whilst network pharmacology, polypharmacy and identification of the right combinations of targets offer major challenges to drug development, it seems critically important that the industry actively explores this area of opportunity and

does not continue to put almost all its efforts into the "one target, one disease" paradigm that may itself be a fundamental cause of low productivity.

3.5 Technology Gaps and the Target Validation Bottleneck

The mismatch that occurred in the early years of genomics between the growth in capabilities in expression profiling and the low capacity and speed of methods for analysing gene function became known as the "target validation bottleneck". This was often exacerbated by the lack of predictive or well-characterised in vitro and in vivo efficacy models. From the description in Sect. 3.5.1 of the functional validation technologies and biological test systems used around 10 years ago, it is clear to see that functional data can often be error prone, equivocal or absent. It is salutary that workers at Bayer found that only 30% of literature reports of positive validation data could be repeated [27], and this agrees with this author's own experience over a number of years which underlines the difficulties in generating reliable functional data (and the risk in depending purely on literature reports). The difficulties in generating unequivocal functional validation data in preclinical studies are likely to have contributed to the failure to filter out many weaker targets. Once a project is established, there can be considerable momentum generated to capitalise on the patentable lead molecules produced in medicinal chemistry, and it can be hard to overturn early interest in a target without definitive negative data as any positive lines of evidence can tend to dominate thinking. Because of the cycle times in drug development, targets that were entering the pipeline 10 years ago are contributing to the poor statistics in Phase 2 and 3 at the present day.

Technological strides in the last decade including gene silencing by RNA interference should contribute to achieving greater confidence in preclinical functional data (see Sects. 9.1–9.5).

3.5.1 Technologies for Modulating Target Activity

Whilst a number of alternative functional validation approaches were available around 2000, they tended to suffer from low throughput, restricted availability, and low success rate in probe generation or could result in artefacts. For example, success in generating neutralising monoclonal antibodies is low by classical methods (less than 10%), dominant negative mutants need careful use due to potential for signalling artefacts and antisense design, and chemistries were usually proprietary to certain biotech companies. Whilst the drug discovery process can generate lead small molecules or biologicals that are well-characterised functional validation probes for use in vivo, these are only achieved by around lead optimisation phase of drug discovery after considerable effort. Unfortunately use of weak, poorly selective or uncharacterised probes at high concentrations was, and is, quite common in publications on target function leading to false positive results.

Gene knockouts in mice (KOs) or transgenics (where a gene is inserted into mice or other species) have become widely used to probe gene function, and initiatives are underway to generate KO mice for all potential druggable targets [28]. However the phenotypic changes are not always predictive of observations in man [29]. A good example of agreement is myostatin which inhibits myogenesis and where the KO in mouse and naturally occurring mutations in cattle and man all result in a major increase in muscle mass and strength [30]. However PPARγ (peroxisome proliferator-activated protein gamma) KO and an agonist both increase insulin sensitivity rather than having opposing effects [31]. Analyses of the available knockouts for the targets of the 100 bestselling drugs [32] and 100 drug pipeline projects in Phase 2 or later [33] showed a high correlation of the phenotype in animal models to the effect of the medicine in man. However, as efficacy in animal models will have been an important criterion for progression of many of these targets into the clinic, a high correlation is perhaps to be expected. Only about 10% of KOs show an overt change of phenotype compared to the wild type under normal conditions. Constitutive deletion or expression may result in physiological adaptation that masks the phenotypic change. Using knockouts in efficacy models can increase the percentage that show an effect, but the value of this depends on the predictivity of the efficacy models (see Sect. 3.5.2.).

3.5.2 Experimental Test Systems In Vitro and In Vivo

In a few indications such as blood cholesterol lowering and β2-adrenoreceptor agonists for asthma where medicines have already reached the market, there is evidence for the translation of efficacy in preclinical models into efficacy in patients. However this clearly cannot be said for first-in-class projects which represent an increasing proportion of drug discovery portfolios.

In vitro monolayers of transformed cell lines have been used widely in both oncology and other diseases often without in-depth studies of their relevance. Cancer patients present with diverse genomic and epigenetic driver changes, and often in the past there has not been an effort to select cell lines with similar genomic changes. Also selective pressures in culture have resulted in cancer cell line acquiring genomic changes not found in primary tumour samples [34, 35]. Cancer cell behaviour and drug sensitivity is dependent on the physical and cellular microenvironment including interactions with other tumour cells, surrounding stromal cells and extracellular matrix which is not modelled by simple monolayer culture. Beyond oncology the use of rapidly dividing transformed cell lines to model, for example, cells involved in inflammation that are terminally differentiated is likely to be even less representative. New methodologies to better model the phenotype and tissue context of normal and disease cells and greater use of primary patient material are discussed in Sect. 9.4.1.

Animal efficacy models offer the potential to evaluate the pivotal nature of a target's function when integrated into in vivo pathophysiology including additional and competing disease mechanisms. Demonstrating robust effects in an animal

efficacy model has been widely seen as essential for project progression. However the target biology is often different in humans and animal model species; e.g. IL8 can act through two chemokine receptors in humans, CXCR1 and 2, which have different distribution and ligand specificities, whereas a single receptor is present in mouse [36].

Animal efficacy models are rarely true disease models and at best can represent some features or mechanisms of human disease. This is well illustrated by animal models of rheumatoid arthritis (RA) which have different dependencies on cytokines, antibodies and inflammatory cells in the initiation and chronic phases and manifest different pathologies and drug sensitivities. The widely used murine collagen-induced arthritis model does not exhibit remissions and exacerbations as occur in RA but is sensitive to therapeutic treatment with anti-TNF agents and methotrexate, whilst the murine streptococcal cell wall model can show exacerbations, but TNFα plays only a very limited role. Arthritis models have played an important part in supporting the progression to the clinic of successful therapies such as the anti-TNFs, tocilizumab (an IL6 receptor blocking antibody) and abatacept (a CD80 and CD86 blocker). Even so the degree of predictivity of any one model can be highly uncertain. Widely used arthritis models are strongly dependent on IL1β, but clinical trials with the IL1 receptor antagonist Anakinra and an IL1 receptor blocking antibody have shown weak efficacy in the clinic [37].

In some area such as neuroprotective agents, the failure rate for translation of efficacy in animal models into the clinic has been 100% [38]. Pain models have a similarly high failure. Whilst arguments have been made that this is the result of poor methodology and animal model design [39], others have argued that each species represents a divergent, evolved complex system that cannot be expected to be predictive of each other [38]. The lessons of personalised medicine and different responses to drugs by individuals, ethnic groups and even between homozygotic twins further underline the ability of even very similar complex systems to behave very differently. Negative results are rarely published making it hard to properly assess the predictivity of animal efficacy models.

Clearly blanket use of a single animal model as a Go, No Go tollgate for target progression decisions prior to the clinic has resulted in false positives and false negatives and will have been a contributory factor to clinical failure. The decision on whether to use an animal efficacy model and what weight to put on the results is clearly challenging. This and recent developments in animal models including genetically engineered mouse models (GEMMs) that model genomic changes in cancers and other human disease are discussed in Sect. 9.4.3.

3.6 Human Genetics

It has been known for a long time that many common diseases such as asthma and diabetes are to a degree inherited, and attempts have been made to identify the underlying genetic factors responsible. In rare genetic disorders such as cystic

fibrosis mutations in a single gene have a profound impact and very high correlation with the manifestation of symptoms (high penetrance) and have been identified by genotyping of affected and unaffected family members. This is done using linkage analysis which depends as a starting point on the fact that the closer an influential allele is to a known chromosomal marker, the more likely it is to segregate with it and not be separated by recombination events (termed linkage disequilibrium). However in many common disease states, heritability has been found to be due to the summation of the effect of multiple, commonly occurring allelic variations each having a small effect (low penetrance) which has become known as the common disease, common variant hypothesis. The search for such genetic variations has been the focus of much effort in order to identify new targets and potential mechanisms in disease [40]. However these studies were often long and costly without giving rise to an output that was unequivocal and exploitable. Common alleles that influence disease susceptibility or resistance are hard to find by family-based studies and require big population-based studies [41]. Because of the low resolution of chromosome mapping available in 2000, it was usually only possible to implicate large stretch of DNA containing many genes, and it required much further work involving sequencing and discovery of new markers (singe nucleotide polymorphisms or SNPs) within this region to home in on the disease-related gene which then requires further functional validation. Also the target may not itself be druggable resulting in pathway analysis to find an alternative. In addition it is not known to what extent the reason for low penetrance is due to a low effect of the allele on target function or if the target itself has a marginal effect.

However the latest developments in SNP identification and of technologies for genome-wide association studies (GWAS) have transformed the ease of human population genetic studies and their use in target identification and validation (see Sect. 9.5).

An alternative to this scanning of the whole genome for susceptibility or resistance genes is to interrogate known deletions or SNPs in a candidate gene for association with disease. All genetic studies depend for their success on how the patient and control cohort are defined and on careful statistical analysis of the results. An example that shows both the value and also the risks of attrition comes from studies of the Δ32 N-terminal deletion in the CCR5 chemokine receptor that was reported to be associated with resistance to both HIV and RA [42, 43]. CCR5 is expressed on the cell surface of CD4+ T cells and inflammatory and antigen-presenting cells and was proposed to be important for their recruitment in arthritis and was also found to be the coreceptor for cell entry for most HIV strains. In the case of HIV, this discovery has led to the development and approval of Maraviroc, a CCR5 antagonist for the treatment of CCR5-trophic strains of HIV [44]. However clinical trials of Maraviroc and two other CCR5 receptor antagonists in rheumatoid arthritis showed no efficacy [45, 46]. Subsequently more highly powered studies and meta-analysis cast doubt on the strength of the initial genetic association with resistance to RA [47].

3.7 Challenges of Clinical Trial Design

Clinical trial design for first-in-class products suffers from many uncertainties, risks and constraints that impact on how well the hypothesis for efficacy can be tested. These include uncertainty about study duration, choice of appropriate responder patient subsets, disease severity and uncertainties about patient numbers and statistical powering. The relatively limited number of regulatory recognised primary end points may mean that there is not an end point that is ideal for testing the new hypothesis. For example, in COPD the lung function tests of forced expiratory volume in 1 second (FEV1) and spirometry are the well-recognised quantitative primary end points [48]. However, for a first-in-class protease inhibitor, the quantitative and temporal correlations between inhibition of the target, improvement in a related pathology end point (e.g. imaging of protection against airspace enlargement) and the primary end point (a significant measurable protection against further fall in FEV1) are likely to be uncertain and difficult to predict.

The importance of patient selection in avoiding false negative outcomes in clinical trials is demonstrated by the EGFR inhibitor gefitinib where a Phase 3 study in advanced non-small cell lung cancer showed no efficacy [49]. However subsequent studies and retrospective analyses have clearly established benefit in progression-free survival in a subset of patients having EGF receptor activating mutations [50]. In many areas of unmet need, such as osteoarthritis (OA), where there is little precedent for positive clinical trial outcomes and underlying disease mechanisms are heterogeneous, patient segmentation, selection of efficacy biomarkers and definition of quantitative end points are still ongoing challenges [51]. Clinical trial design in Alzheimer Disease regards at what stage to treat and for what duration has only slowly progressed through a process of experimentation over the last twenty years inevitably resulting in suboptimal testing of many drugs in the past [52].

Successful clinical trial design requires good integration of preclinical and clinical knowledge and communication across disciplines which is frequently difficult to achieve and maintain over the long drug discovery cycle times. Also there are likely to be cost considerations to factor in that might impact on decisions about design.

Whilst much discussion has focused on target selection and preclinical target validation as a source of project failure, it is likely that clinical trial design can be a major reason also. The recent focus on patient stratification and personalised medicine involving molecular, genetic and clinical characterisation of patient segments, development of new biomarkers and more extensive co-operation between regulators and pharmaceutical companies should result in improved clinical trial design (see Sects. 11 and 12).

3.8 Target-Related Safety Liabilities

It has been estimated that overall drug compound attrition rates for reasons of safety are currently 30% and from analysis of projects at Bristol-Myers Squibb and Merck

between 1993 and 2006 28%, or this was due to target-related pharmacology [53]. Therefore assessment of the role of the target in normal physiology and its potential safety liabilities is an important target validation issue. The importance to patients and the success of the industry itself of correctly assessing safety risks versus benefits was graphically demonstrated by the withdrawal of Merck's selective cyclooxygenase-2 inhibitor Vioxx [54].

Advances in in vitro predictive toxicology methods and toxicogenomics are likely to play an increasing role in early identification and elucidation of the mechanism of target-based adverse effects in the future (see Sect. 10).

4 Strategies for Reducing Attrition

The urgent need to improve prediction of clinical efficacy and drug discovery productivity has generated a vigorous response to many of these root causes amongst scientists and management in the pharmaceutical sector.

One direction has been to focus on improving the efficient management of each step in drug discovery, reducing cycle time and the management of pipeline metrics in order to reduce cost and time and achieve more "shots on goal" in the clinic. This has included a growing application of process management methodologies for quality control, waste elimination and process optimisation such as Lean Six Sigma in companies such as Pfizer and AstraZeneca [55, 56]. Whilst these process improvements may result in useful incremental improvements in the cost-effectiveness of drug discovery, they are being applied to what is in most cases an already well-optimised process and do not address the core underlying waste which is the cost of failed Phase 2 and 3 clinical trials due to poor efficacy.

Developments in drug discovery research that may start to address the root causes explored here are reviewed in Sects. 5–12. The learning from the author's own experience in early target selection and preclinical target progression is described in Sect. 13.

5 Having the Right Product Vision and Delivering Commercial Success

It is possible to take a purely target-led approach to arrive at a target product profile (TPP) in which the biology of a druggable target is profiled to see if it is likely to meet any known patient need. The alternative is to have a clear product vision at the outset crystallised from investigations of unmet medical need and seek targets that will deliver this TPP. Both approaches have been used, but the latter allows effort to be focused on setting up test systems for evaluating and comparing the importance of a number of candidate targets in cell responses, disease processes and

pathologies relevant to the unmet need. These biological tests can be time-consuming and expensive to develop and validate making a focus on a defined patient need more efficient as well as being more likely to align with a good commercial opportunity. This patient-led approach requires collaborative working and clear communication between preclinical molecular, cell and in vivo biologists, clinicians and those involved in assessments of the commercial opportunity.

The assessment of the commercial viability of a TPP is a challenging activity but important in reducing the risk of a newly approved medicine failing at the last hurdle. These analyses are done by most pharmaceutical companies and should take into account factors such as the size of the patient population or segment, the estimated market share given other competitors likely to be on the market at the time of launch, and the price payers may be willing to pay for the predicted improvement in patient health.

Payers such as the National Health Service use their advisory body, the National Institute for Health and Care Excellence (NICE), to undertake cost-effectiveness analyses to guide their decision on whether to recommend prescribing a newly approved medicine. The most widely used basic unit for this analysis is the QALY (quality adjusted life year) which is the expected years of life remaining multiplied by a utility score representing the level of the patient's health. The cost-effectiveness is estimated by calculating the increased cost of a new treatment per QUALY gained by its use (£ per QALY) [57] also known as the incremental cost-effectiveness ratio (ICER). NICE and the Scottish Medicine Consortium set threshold levels based on the overall financial imperative of maximising the value derived from the available health-care budget such that a medicine is generally considered cost-effective only if the £/QALY estimate is below 20–30,000. It is widely recognised that this analysis has both theoretical and methodological weaknesses. However alternative approaches such as estimating willingness to pay (WTP) per QALY have proved very complex and given widely varying results, and to date there is not as a consensus about an improved approach [58, 59].

This has become a contentious area as it sometimes blocks patient access to new medicines that may have shown benefit in clinical trials. Also it puts a largely economic tollgate at the end of many years of work developing a new and effective medicine. Therefore it is essential that drug companies engage proactively with the assessment of cost-effectiveness and take measures to provide the best supporting evidence. Carefully controlled Phase 3 studies often do not fully reflect experience in clinical practice, as shown for etanercept [60], or form a good basis for QALY calculations. So Phase 4 or "naturalised" in life trials can also be important for pharmaceutical companies to provide adequate supporting evidence to public and private payers.

The importance of setting an appropriate price is demonstrated by abatacept (Orencia), a fusion protein that blocks T cell co-stimulation for use in RA. Initial NICE guidance was to restrict use to late-stage disease when two non-biological disease-modifying treatments (DMARDS), anti-TNF therapy and the anti-B cell antibody rituximab had all failed. Discounting Orencia through a patient access scheme by Bristol-Myers Squibb has led NICE to reappraise its guidance and recommend it as an option for use as an alternative to other biologicals [61].

5.1 A Successful Example from Asthma

The example of the development by Novartis of omalizumab (Xolair), a monoclonal antibody that neutralises IgE, is a useful example to illustrate the importance in drug development of understanding patient need, commercial opportunity, correct clinical study design and effective interaction with those assessing cost-effectiveness.

Asthma is characterised in the clinic by reversible, bronchoconstriction with mucus production and underlying inflammation resulting from an increased sensitivity to specific allergens (and other agents, e.g. smoke particulates). Mild intermittent and mild or moderate persistent asthma are usually controlled well (but not fully) by regular use of cost-effective inhaled long-acting β2-adrenoreceptor agonist bronchodilators with inhaled corticosteroids plus the addition of leukotriene receptor antagonists as an "add-on" therapy as required. However there are a small number of patients who have severe disease and are much less responsive to steroid treatment (including oral) and whose condition is unstable resulting in exacerbations that frequently require hospitalisation and can result in death. Only about half these severe asthmatics have high circulating IgE levels and other hallmarks of allergy. Unstable asthma accounts for the majority of the health-care burden largely due to the cost of hospitalisation during exacerbations.

Many possible product profiles present themselves from this overview that could meet patient needs including a new, more effective, oral, first-line therapy for symptom relief in mild and moderate asthmatics at a similar cost per patient as inhaled treatments. Xolair as a monoclonal antibody would have a high cost per patient per year, so the challenge of demonstrating good cost-effectiveness was particularly great. Also the treatment requires attendance at the clinic for i.v. infusion so is only applicable to more severe disease. Therefore focusing the clinical trials on severe asthmatics and choosing exacerbation frequency as the primary end point was important in clinical development. In the Phase 3 INNOVATE study, Xolair was successful as an add-on therapy in improving lung function, quality of life measures and reducing likelihood of exacerbations [62]. Even so arriving at an acceptable ICER required post-study analysis and identification of those with a prior history of frequent exacerbations as the group most likely to benefit. Achieving agreement that reduced mortality could be predicted from reduced exacerbations had a strong influence on whether Xolair treatment would give an acceptable but borderline ICER in the NICE and other evaluations. In the case of Xolair, a 1-year "in life" open-label Phase 4 study (ETOPA) showed that longer treatment in this setting resulted in an improved ICER [63]. Xolair was recommended by NICE for use though only in severe asthmatics where other therapies had failed and dependence on allergen and IgE could be demonstrated and where there was a history of frequent exacerbations. Subsequently cost-effectiveness analyses in several countries have further confirmed the cost-effectiveness of Xolair and led to the recommendation by NICE for use in children if using oral steroids frequently [64, 65].

The above discussion shows the risks to commercial success of a newly approved medicine and the uncertainties inherent in decisions of cost-effectiveness. It is very important that drug companies do all that is possible to reduce risks in these areas and ensure correct positioning, trial design including "in life" studies and pricing to build the case for cost-effectiveness.

6 Improved Disease Research: The Importance of Collaboration and Open Access

Detailed knowledge of disease mechanisms is clearly critical for target selection and validation as well as correct design of clinical trials including selection of end points and biomarkers. This involves understanding the linkage between target function and primary end points in terms of intracellular signal transduction, cell interactions and their relationship to observed pathologies. One of the consequences of poor success in the clinic has been the recognition by the pharmaceutical sector of the need for a greater understanding of disease processes and how these vary between patients within the same disease. Over the last 10 years, there has been impressive progress in the description of the disease processes at the genomic, epigenetic, transcriptional, cell signalling, tissue and whole-body levels and in the development of technologies to study them. (Many of these technologies are also central to generation of target validation data and are described in Sect. 9.) However this growth of information has been slow to translate into a greater clarity about how to combat disease and pick winning targets.

An important response to this failure to understand the underlying driver versus bystander events in common diseases (and many other large drug discovery challenges) has been a growing conviction that all the necessary leading science and innovative thinking cannot be found inside any one company alone. This has given new impetus to collaborative and open access forms of working and to looking outside to a greater extent for transformational innovations, disease understanding and new project concepts. Also the failure to improve the flow of new medicines has resulted in central government increasing research funding on collaborative projects and programmes such as the FDA's Critical Path (c-Path) Initiative and the EU's Euro 2 billion Innovative Medicine Initiative (IMI) both of which aim to promote innovations in health care through creating public private partnerships between companies and institutions with leading expertise and capabilities. Table 1 shows just a small number of recent examples of the wide range of collaborations established to develop enabling technologies, biological assay systems, elucidate target function or disease mechanisms and discover biomarkers and strategies for personalised medicine. Table 1 also illustrates the diversity of organisational structures employed.

The potential benefits of collaborative working are many. For early initiatives such as the Biomarker Consortium and c-Path's Predictive Safety Test Consortium

Table 1 Examples of collaborative and open access working and different partnering models

Scope	Collaboration	Description and objectives
Enabling technology	SAGE Bionetworks http://sagebase.org/about-2/	Open access collaboration of large pharma and academia to develop novel IT platforms to analyse biomedical data, create disease models and predict health outcomes
	Structural Genomics Consortium http://www.thesgc.org/	Open access consortium (grant bodies and 8 pharma) for HT-protein crystal structures and tools for functional studies
	Harvard Stem Cell Institute http://www.hsci.harvard.edu/	Academic institute collaboration with PPP to advance stem cells uses in medicine
Target identification	Cenix and AstraZeneca and other pharma http://www.cenix.com/news/	Closed contract research between pharma and a technology provider
	Grants4Targets http://www.grants4targets.com/	Bayer grants and technology support (e.g. antibodies) to applicants for validation of targets
	Senectus (Cancer UK start-up) http://www.senectustherapeutics.co.uk/news/	Discovery of oncology targets in senescence using AZ compound bank and Senectus screens
Disease mechanisms	IMIDIA http://www.imi.europa.eu/content/imidia	EU (IMI) funded PPP to improve β-cell function and diabetes diagnostic biomarkers (8 large pharma, 12 universities and research centres)
	Insulin Resistance Pathway project http://www2.smartbrief.com/news/aaaa/industryBW-detail.jsp?id=1ECE4C29-826D-4383-B5B7-57967A349657	PPP collaboration (Pfizer, 4 US universities, Entelos) to understand diabetes and obesity mechanisms in adipose cells including the use computational biology
In vitro and in vivo models	PREDECT http://www.predect.eu/about/	EU (IMI) funded PPP for improved predictive 3D and coculture models and models in vivo, for TV in oncology (9 pharma, 9 universities and institutes, 3 SMEs)
	Consortium lead by P1vital (CRO in CNS clinical trials) http://www.p1vital.com/consortium.html	Initiated by P1vital linking its academic network and 5 pharma to establish in man experimental models of CNS disorders
Personalised medicine and biomarkers	Biomarker Consortium http://www.biomarkersconsortium.org/	Open access consortium (NIH, FDA, 14 pharma, 16 public institutes) to identify and validate biomarkers and gain regulatory acceptance
	OncoTrack http://www.oncotrack.eu/	EU (IMI) funded PPP to identify novel colon cancer biomarkers for personalised medicine (8 pharma, 7 universities and institutes, 4 SME)

Predictive safety	Predictive Safety Test Consortium http://c-path.org/programs/pstc/	Collaboration (FDA, EMA, Japan and 19 pharma) to validate safety tests as part of C-Path Initiative to lower drug attrition and timelines
Encouraging innovation	Centres for Therapeutic Innovation http://www.pfizer.com/research/rd_works/centers_for_therapeutic_innovation	Pfizer funded joint labs in medical academic centres to speed translation of early drug discovery ideas to the clinic with access to Pfizer biologics and compounds for TV
	Bayer CoLaborator hub in San Francisco http://www.bayerpharma.com/en/partnering/news/news-page.php/14705/2012-0396	Incubator labs with close working and Bayer capabilities for start-ups aligned to Bayer objectives
	Enlight Biosciences LLC http://www.enlightbio.com/portfolio-companies.php	A syndicate (PureTech Ventures and 7 pharma) to invest in start-ups with transformational technologies in health care

(Table 1), in which most large pharmaceutical companies are involved, the need to bring together adequate amounts of validation data and standardise methodologies to assist regulatory acceptance whilst sharing costs are clear advantages. The pharmaceutical industry has a long track record in establishing alliances with biotech companies with complementary capabilities to advance their projects. Between 1997 and 2002 large pharma entered into approximately 1,500 such alliances [66]. Also the pharmaceutical industry traditionally has sought collaborations with academic institutions. However, in the past there was often a mismatch of objectives and cultures between the industrial partner (tending to be project-, problem- and timeline-focused and protective of IP) and their academic partners (tending to be focused on knowledge generation and dissemination) resulting in suboptimal collaborations and low knowledge sharing. Lately the recognition of the need to understand fundamental disease biology in greater depth has resulted in broader, long-term collaborations involving more partners and varying degrees of open access to the wider research community such as the Pfizer's $14 M Insulin Resistance Pathways Project. Such initiatives may better align the objectives of the partners [66]. The larger numbers of partners in such collaboration from a range of disciplines will hopefully achieve a more complete and integrated output. However these forms of consortia and partnerships with wide scope and multiple partner institutions can consume high levels of research funding and present a much greater challenge to efficient management, so it still remains to be seen how many will prove truly cost-effective in delivering health-care innovation.

Pharmaceutical companies are also investing in translational science initiatives and early identification of promising innovations in academia and start-ups by funding public private laboratories integrating pharma and academic staff such as Bayer's CoLaborator and Pfizer's Centers for Therapeutic Innovation. Several companies are also providing venture capital support for transformational developments in health care through such means as Enlight (Table 1).

There is a growing willingness in large pharmaceutical companies to overcome traditional concerns about loss of confidentiality and intellectual property rights and work collaboratively and precompetitively in open innovation initiatives where all biological information is made freely available to the wider research community. A powerful example of a fully open, IP-free initiative is the Structural Genomics Consortium (SGC) based in Oxford, Toronto, and Karolinska Universities and funded by several grant authorities and pharmaceutical companies. Its aim is high-throughput X-ray crystal structure analysis of druggable target classes and tools for exploring target function such as antibodies and chemical probes [67]. The SGC has so far contributed 1,400 structures to the Protein Data Bank; information that would previously have been considered proprietary because of its value in facilitating drug design. The SCG has a powerful (more than 200) network of academic collaborators that can quickly, without IP barriers, generate open access, biological information using these proteins and probes.

The value to successful target selection of open access for all information on human disease biology and target function is obvious. This argues for a culture of enlightened self-interest where all target validation information and disease biology

is considered precompetitive and shared as there is still ample opportunity for companies to pursue proprietary drug discovery projects based on how such information is used to define TPPs and the medicinal chemistry strategy they adopt.

Clinical trial information, whether positive or negative, can feed back much useful information about disease mechanisms and suitability of biomarkers. So it is important that outcomes are made known and reported accurately. As a result of concerns about reporting of adverse events and interpretation of efficacy data, there has been a concerted move in this direction by regulatory bodies such as the FDA and EMA, industry bodies such as EFPIA and companies themselves to ensure higher levels of transparency [68]. This has included legislation in the USA. Glaxosmithkline recently pledged to establish a process by which all patient level data can be made available to researches with valid scientific questions.

Patient health records are increasingly being seen as an underused resource for biomedical research as they contain data and test results on progression, response to treatment and occurrence of comorbidities. The growth in the existence of electronic health records (EHRs) has opened up the possibility to mine these data to identify new associations between disease and patient phenotypes and genotypes [69, 70] and new approaches to patient stratification and personalised medicine. Currently much effort is going into overcoming the issues of ensuring anonymity, developing consistent terminologies for use across health-care organisations and bioinformatic tools to adequately integrate and mine the diverse types of data. Public private partnerships such as the IMI's Electronic Health Records for Clinical Research (ECR4CR) aim to facilitate this process [71]. An early example of the potential utility of ECRs has come from integration of genome-wide scan data and ECRs leading to the identification of a previously unknown linkage between SNPs in the FOXE1 region with primary hypothyroidism and other thyroid disorders [72].

This new area of clinical and medical informatics is generating expectations of transformational discoveries and efficiencies through the ability to probe the same ECR datasets with different questions and select different cohorts for different purposes. However it is important to keep in mind the lessons from genomics that large amounts of data do not automatically translate into improved understanding of disease, so it is important that investment is linked to, and directed by, positive outcomes from early initiatives.

7 Clear Definition of the Hypothesis Linking the Target to Unmet Patient Need

A crucial step in target identification and validation thinking is the creation of the hypothesis describing the molecular and cellular mechanisms by which a target initiates or causes progression of the disease (or if the target is not directly part of the disease process, then how pharmacological modulation of its activity will influence disease mechanisms in a beneficial way). It is important to recognise

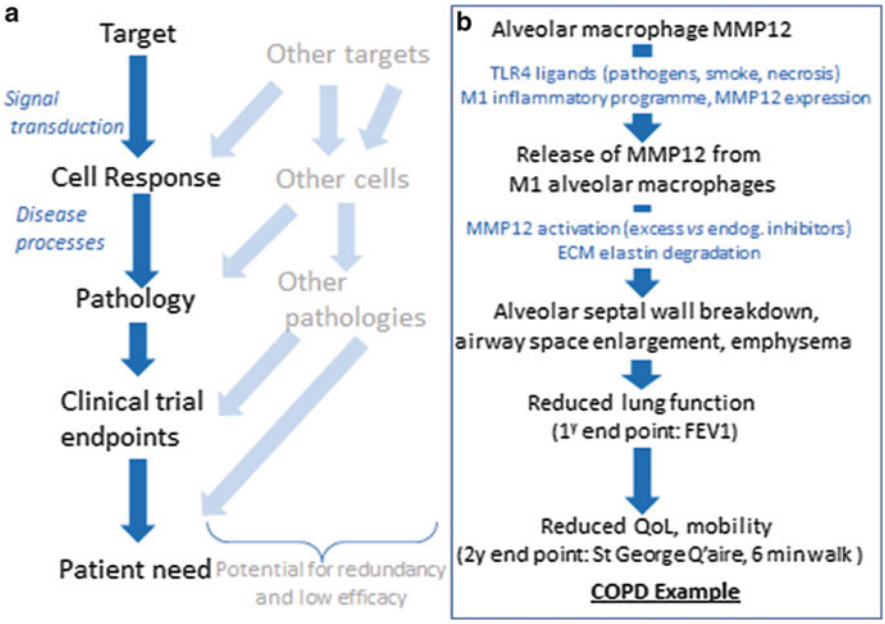

Fig. 1 Steps in the hypothesis linking the target to patient need. Generic causative steps used in the description of the hypothesis for target efficacy are shown in (**a**) along with the potential for low efficacy due to target redundancy and contributions from additional disease mechanisms (described further in Sect. 7). (**b**) The causative steps are exemplified for MMP12 which could be considered as a candidate for target selection on the basis of [73, 74]

that this hypothesis for efficacy usually comprised a series of causative steps linking different levels of biological organisation (the target, the cell, the tissue and the patient), and each is itself a hypothesis. Firstly at the intracellular level a change in the target activity arising from, for example, mutation, expression or changes in cellular location is postulated to give rise to a change in cell behaviour. Observed changes in cell behaviour in disease such as migration, death, division or mediator release are hypothesised to underlie disease processes that in turn are postulated to give rise to the observed pathologies. Finally, at the highest level these observed pathologies, manifested as structural and physiological changes, are hypothesised to give rise to the symptoms and unmet patient need. These steps are shown in Fig. 1 and are similar to those described by others [75]. Whilst this may seem obvious, it is important to consider the value, limitations and consequences of this approach.

Firstly, the component hypothesis for each causative step may be more or less correct, and validation evidence needs to be evaluated or generated for each. Clearly if there is even a low or moderate risk associated with each sub-hypothesis, then this can multiply up to a high risk overall. Evaluating the strength of existing evidence for each component hypothesis also facilitates assessment of overall risk and allows the focus of resource on addressing the high-risk gaps. The strength of the component hypothesis linking an observed pathology and

the unmet patient need is frequently an area of unrecognised high risk. An example that illustrates this is the unexpected result that reduction in blood and sputum eosinophils by the anti-interleukin-5 monoclonal antibody mepolizumab did not reduce responsiveness to antigen challenge or improve lung function in moderate persistent asthmatics despite the evidence that IL5 was important for eosinophil maturation and activation and the role attributed to eosinophils in bronchoconstriction and lung inflammation [76]. Subsequent discussions centred around whether lumen or tissue eosinophil number had been adequately suppressed underlining the uncertainty that can exist in the quantitative relationship between cell responses, pathology and symptoms. Recognising uncertainty and risk in the hypothesis at this level and undertaking studies to characterise these linkages is an important aspect of increasing our understanding of disease.

A target may be active in more than one cell or pathological process; e.g. neutrophil elastase could be analysed in this way for loss of lung extracellular matrix and consequences in emphysema [77] but also for increased mucus production and its consequences in bronchitis [78]. It can be valuable to analyse the validation evidence for more than one causal chain of events in order to establish which is the stronger hypothesis overall or to identify the likelihood of additional sources of efficacy. Such analyses can influence important decisions, e.g. the choice between bronchitis or emphysema patients or both in clinical trial design for neutrophil elastase.

However it must be recognised that a simple linear description of the causative links between target and unmet need is usually an oversimplification (see Fig. 1). The patient need is likely to be the result of several pathologies, and each will be affected by many disease processes involving different cells. For the pathology of airway space enlargement in the MMP12 example in Fig. 1, some of the other disease processes involved are likely to be inflammatory cell influx, increased endothelial and epithelial cell apoptosis and failure of matrix repair in addition to increased extracellular matrix degradation [75]. Each of these processes will involve several cell types containing several potential targets. There can be substantial redundancy at the target level; e.g. several proteases in the COPD lung, including MMP12 and neutrophil elastase, have overlapping substrate specificities and could be more or less important in extracellular matrix breakdown [79]. In target selection it is important to take into account all that is known about this broader context of contributing disease processes and potential target redundancy in order to minimise the chance of poor efficacy. Also in the validation strategy it may be necessary to design studies to probe the importance of these competing mechanisms or targets.

Also diseases tend to involve positive and negative feedback loops and frequently involve the whole body; e.g. inflammatory disease in a particular tissue also involves increased bone marrow production of progenitors and the transport of inflammatory cells through the blood. In reality the disease state may best be described as a complex interaction network. Systems biology aspires to model and understand the regulation of the “interactome” of the disease state more fully in the future.

In the meantime the description of the hypothesis for efficacy in terms of a linear series of causative steps linking the target to patient need still provides a useful practical framework for target validation studies and has the advantage of subdividing the overall hypothesis into a number of steps each of which tends to be suitable for experimental testing. In this it is extremely important to define the component hypotheses in adequate detail in order to design experiments and biology test systems that ask the correct validation questions. For the example given in Fig. 1, rather than stating the hypothesis as "MMP12 from inflammatory cells causes lung tissue damage", it is more valuable to formulate this as "MMP12, released from M1 subtype of alveolar macrophages, is activated outside the cell and degrades septal wall basement membrane elastin". This more precise description allows specific experiments to be designed to confirm the cell of origin, extracellular activation, substrate specificity and tissue localisation. Sections 9.1–9.5 describe the types of preclinical evidence and techniques that can be marshalled to derive reliable evidence to support the target playing a major role at each organisational level.

If sound evidence can be obtained that a target has a profound and nonredundant effect at the level of cell response, disease process and pathology consistent with the hypothesis, then it is likely to provide a good preclinical platform for progression. Importantly if during this analysis evidence arises that is clearly discordant with one or more component hypotheses, this should halt progression at least until the implications are properly investigated.

8 Where Do Targets Come from?

Certain trends in target selection have tended to dominate at different times as was described in Sect. 3.4. Now companies use multiple strategies to try to identify the best targets and build a diverse portfolio with a spread of novelty and risk. However for most targets currently populating the pharmaceutical company pipelines, public domain information is likely to have been the starting point. Because everyone has access to similar public domain information, there is usually considerable overlap between drug discovery pipelines. However it is interesting to note that the public domain information can lead different companies to align the same target against different indications perhaps for portfolio reasons or different interpretation of the strength of the validation information.

Following the earlier emphasis on expression profiling to identify candidate targets, there has been renewed interest in phenotype-led approaches including screening large libraries with diverse chemistry for compounds with useful properties (sometimes called chemical biology) or collections of known drugs to discover novel indications (known as drug repositioning or repurposing). A major new direction has been functional genomics which involves high-throughput screening of large libraries of reagents such as siRNAs, shRNAs or cDNAs that are designed to probe the function of large sets of genes or the whole genome.

The CRISPR/Cas9 system for gene editing (see Sect. 9.4.4) has begun to be used for genome-wide studies also [80]. Functional genomic studies using siRNA in HIV cell models have indicated that there may be severalfold more potential targets than are known in the literature [81]. These phenotype-led approaches tend to identify more novel targets and make more proprietary observations than the evidence-based approach. Targets and pathways can impact on cell phenotype through mechanisms that are not always obvious or predictable. The analysis of approvals by the FDA over a 10-year period has indicated that phenotypic screening leads to more first-in-class small molecule drugs with new molecular mechanisms of action than target-based approaches [82].

However knowledge-based approaches and functional genomics are not mutually exclusive and can be used in combination; for example, public domain information or expression studies can often be used to prioritise the sometimes large number of hits from functional genomic RNAi screens. More recently developments in systems biology have raised hopes of being able to predict targets by in silico methods in the future.

8.1 Evidence-Based Target Prioritisation

Given that public domain information will continue to make important inputs into target identification and selection, it is important to use the latest bioinformatics and information science strategies including ontogeny-based approaches to bring together all strands of information from such sources as scientific papers, patents, genomic and expression databases, etc. In large pharma it is also important to search and integrate proprietary databases and information sources. It is good practice to decide on a set of target selection criteria to compare a target against (discussed further in Sect. 13.2). When prioritising a number of targets, each is likely to present with a different profile of strengths, weaknesses and gaps, and this will frequently result in the need to fill gaps or confirm pivotal results before target selection. Functional data in relevant biological test systems or strong genetic linkage to disease constitute more influential evidence than expression changes in disease samples. It is important to recognising that scientific literature can result in unintentional bias in target selection. The first member of the mitogen-activated protein (MAP) kinase family, p38α, is the subject of many thousands of papers, and many tools are available for its study, whereas family members discovered later are less well studied, have fewer tools and will look less attractive as a target.

8.2 Functional Genomics and High-Throughput RNAi

Functional genomics has become a powerful new approach in target discovery in the last decade with RNAi becoming the most widely used technology (described further in Sect. 9.4.4). The efficiency of siRNA and shRNA design and synthesis

quickly led to libraries of gene silencing reagents able to target every gene in the genome including regulatory microRNAs. Combination with high-content biology and imaging is commonly used to characterise effects of gene silencing on cell function and also off-target toxicities. High-throughput siRNA screening is often performed using a single oligonucleotide per well or pooled reagents against a single gene. Chemically modified siRNAs are used in the leading libraries to reduce off-target effects [83] discussed further in Sect. 9.4.4. Plasmid- or viral-born shRNA can be screened singly or transfected into cell populations as pools against large sets of genes. In the latter case, a change in cell phenotype is selected for and the active shRNA sequenced to identify the target gene. In such experiments it is very important that a phenotype such as cell survival in vitro or metastasis formation in vivo is used that is clearly distinguishable from off-target toxicities.

HT-RNAi can prove a highly valuable approach for discovering novel targets. A good example is the identification of synthetic lethal combinations that would not have been predictable on the basis of knowledge of cell signalling alone [84]. It is being applied widely in many settings particularly in oncology including cell lines [85], primary cells [86] and cells treated with RNAi ex vivo and adoptively transferred in vivo [87].

However this remains a very demanding technology requiring considerable care in assay validation, [88] and concern has been raised about the apparent lack of agreement across laboratories [89]. Best practice in the field would be encouraged by the existence of minimum reporting guidelines for publications as have been developed for gene chip expression studies. Use of well-designed positive and negative controls is important [90] as is hit confirmation through repeat screening and assessing the degree of concordance between multiple independent RNAi reagents. Due to the number of hits, it is usually necessary to devise a series of criteria to prioritise the most suitable targets such as whether there is clustering in certain pathways. Interestingly "Haystack Analysis" of the seed sequence of siRNAs having their effect on phenotype via off-target effects may be useful in identifying microRNAs that also regulate the cell response [91].

In applying HT-RNAi it needs to be born in mind that it may take 6–12 months to arrive at a prioritised hit list. Also, as a key value of functional genomics is to identify novel targets, then it may be necessary to invest greater internal effort to build a validation data package to meet pipeline entry criteria for a novel target than for a literature target.

8.3 Genome-Wide Association Studies

The International HapMap Consortium [92] has identified more than 1 million SNPs that occur with a frequency of 1% or more in the genomes of several major ethnic groups. Commercial hybridisation arrays for the 500,000 SNPs that represent the most common haplotypes in the human genome have been developed, and these have made genome-wide association studies (GWAS) a much more rapid and cost-

effective tool for hypothesis-free target identification than in the past [41]. However because of linkage disequilibrium the influential gene still needs to be investigated further as the analysis usually still only identifies that it is within small region of DNA. Such studies are rapidly expending the breadth of knowledge about susceptibility and resistance genes, e.g. in asthma [93], and opening up new approaches for patient stratification. There is a need for best practice in the use of the hybridisation chips for SNP analysis as well as careful definition and validation of patient and control groups and statistical analysis [41]. Next-generation sequencing methods are likely to extend this approach to more SNPs in the genome.

8.4 Chemistry-Led Target Identification

The random screening of libraries for compounds that have the desired effect or identifying them by literature mining or chance observation can also result in the discovery of unexpected disease-modifying mechanisms. At AstraZeneca Charnwood it was discovered that a group of immunomodulatory compounds were able to block primary T-cell expansion by an unknown mechanism. This mechanism proved to be block of the export of lactic acid produced as a result of the very high level of metabolic activity of these cells after activation through inhibition of the monocarboxylate transporter 1 (MCT1) [94].

However issues with this approach are that the mechanism of action can prove very demanding to elucidate (as was the case for MCT1). This may involve medicinal chemistry to enhance compound potency and to develop affinity labelled probes. Analysis of interaction partners through various approaches may be required. Finally confirmation of the mode of action through modulation of target function by independent methods such as RNAi will be important. Compounds may mediate their effect through more than one target which again could lead to useful discoveries but also difficulties in elucidating the mode of action. A further risk is that any safety liabilities associated with the mechanism will only become apparent after all this effort has been made.

8.5 Drug Repositioning

Many of the top-selling medicines have found their major application in a different indication than the one for which they were developed, e.g. Viagra [95]. Also it is often the case that targets have different functions in different physiological contexts, and the first function identified may not be the most useful for therapy (as the naming of tumour necrosis factor shows). Given the cost of developing a new candidate drug, if no or little efficacy is shown in the clinic then there is clearly great value in identifying alternative indication. Also the profitability of a launched medicine can be greatly enhanced by identification of a second indication. This has led to many strategies to identify new indications by screening candidate drugs or

other mature chemical assets in silico, in cell systems in vitro or in animal models. There is evidence to suggest that this is a productive approach to achieving new FDA approvals [96].

8.6 Network Pharmacology, Polypharmacy and Target Combinations

As described in Sect. 3.4.3 network theory and much practical experience in disease therapy indicate that disease phenotypes tend to be robust to the removal of an individual component or connections in their protein interaction network making it important for the pharmaceutical industry in the future to concentrate more effort in the area of polypharmacy and discovery of new target combinations.

Combinations that are used in the clinic have tended to become the standard therapy as a result of years of accumulated experience, e.g. the treatment guidelines for asthma. Current initiatives, whilst valuable, almost exclusively involve attempts to combine existing approved medicines either co-administered or co-formulated as in the case of AstraZeneca's Symbicort (an inhaled topical steroid and β-agonist combination). However currently only about 320 targets are exploited by all medicines, and whilst combining these in novel ways may greatly increase their utility, it still represents a very small fraction of the potential of the genome and excludes combinations of targets that may be highly efficacious only when targeted together but silent alone. There are clearly challenges in both the identification of new target pairs de novo and in development of drug combinations, but there is encouragement from a number of directions that these are not insurmountable.

The total number of potential target combinations in the genome is massive and too big to be explored systematically even by RNAi library screening. An obvious strategy is to use a knowledge-based approach where combinations of targets are selected on the basis of current understanding of the pathways and mechanisms of disease. A target identification opportunity that exploits the concept of network pharmacology but reduces the size of the problem is the synthetic lethal approach where one of the interacting pair of targets has been mutated to become an oncogene and so is fixed. An example of this was the finding that poly(ADP-ribose) polymerase (PARP) inhibitors show differential lethality in breast cancer cell line with the BRAC1 and BRAC2 mutations, and a whole genome siRNA synthetic lethal screen revealed further strong enhancers of PARP inhibition [97]. If one target is already selected by, for example, showing some but insufficient efficacy in preclinical or clinical studies, then it is feasible to screen in vitro the whole or the druggable genome by RNAi for enhancers [84].

For regulatory approval safety testing of each pharmacological agent individually and in combination is likely to be needed. Clinical trials may be larger and more complex if there is a requirement to demonstrate the benefit of the combination over monotherapy thus increasing development costs. Given these hurdles there could be an advantage in combining activity against multiple proteins in a single molecule by

identifying pharmacophores with dual activity or tethering separate ligands together. The combined β2-adrenoreceptor agonist and dopamine D2 receptor agonist, Viozan, taken into late development for COPD by AstraZeneca [98] is an example of how the structure activity and pharmacokinetic and pharmacodynamic relationships for both can be optimised and balanced. Cheminformatic analysis and experience from high-throughput screening has shown that pharmacophores and members of low molecular weight fragment libraries can also cross target classes [22].

Large molecule therapeutic platforms offer important opportunities for therapies against multiple targets. The clinical testing of combinations of therapeutic oligonucleotides seems to face less regulatory hurdles (e.g. Alnylam's ALN-VSP, a co-formulation of two siRNAs targeting VEGF and kinesin spindle protein for liver cancer that has completed Phase 1) [99]. Also an area of strong investment is bi-specific or multi-specific antibodies where a single antibody or construct carries different variable regions that bind to different target antigens. There is one launched bi-specific antibody for cancer, catumaxomab, and many others in development [100].

8.7 Systems Biology

Systems biology populates computer models with experimental measurements of the parameters of a biological system in order to understand the structure, dynamics and control mechanisms of the system. It is being applied to model normal and disease states at the pathway, cellular, tissue or whole organism level and aims to predict how these will respond to targeting [101]. Known drug targets are being studied to understand how they relate to the topography of their interaction networks to try to identify characteristics that are most likely to deliver a phenotype change. Initially the nodes with the highest number of interactions were thought to be the best targets, but recent analyses are focusing on where the components are involved in connections between subregions of the network (termed bridging centrality) [102]. However the in silico prediction of the best targets is still at a very early stage.

Systems biology is a rapidly growing area of computational biology and is attracting much investment, for example, the $14 M collaboration in diabetes between Pfizer and universities in California. There have been successful demonstration of application of systems biology in, for example, blood clotting [103]. However, modelling the heterogeneity of most diseases, their progression over time as well as the differences that form the basis for patient segmentation is likely to present a huge challenge. Also many cellular and physiological control mechanisms remain incompletely understood, e.g. microRNAs and other regulatory RNAs. The effort required to generate the experimental data to populate such models could be large, and without an understanding of what level of complexity can be successfully modelled, there is a danger that many studies could be unproductive or not cost-effective.

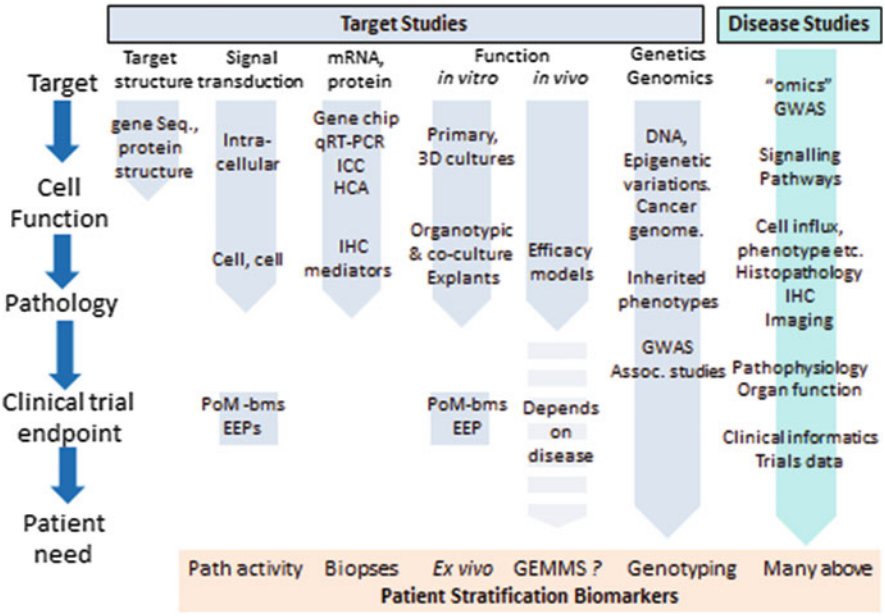

Fig. 2 Sources of evidence for the sub-hypotheses for efficacy. Some of the main ways that the technologies described in Sect. 9 can contribute evidence to support the causative steps linking the target to patient need (as described in Sect. 7) are shown. Research into disease processes also plays an integral part. Many of these approaches can be utilised for patient stratification as shown. (Fig. 2 is of necessity a simplification and is not intended to be comprehensive). Abbreviations not defined in text are bms, biomarkers, and EEPs, exploratory end points

9 Sources of Preclinical Evidence to Support the Hypothesis for Efficacy

How different sources of validation data can provide support for the different causative steps in the hypothesis linking changes in target activity to unmet patient need will be described (see also Fig. 2). For each of the component hypothesis, it is important to draw on more than one independent source of validation data where possible to build a robust platform of evidence. The experimental approaches that can contribute this supporting data are very diverse, and it is not possible to describe them fully here, but some recent reviews are cited. The techniques will be organised under what can be learned from target structure, expression studies, signal transduction, studies of function in biological test systems in vitro and in vivo and from human genetics. Recent developments such as the use of high-content analysis and multiplex assays to give a more complete picture of the role of the target in cellular events will be highlighted as will the value of incorporating primary human patient material into studies.

To reduce the lack of reproducibility of target validation data encountered by Beyer [27] and ourselves, it is important to apply best practice. Therefore there will

be emphasis on careful use and interpretation of the results of these technologies as most have strengths, limitations and the capacity to generate artefacts.

As will be seen, computational biology and bioinformatics in different forms have become an essential and integral part of target validation technologies and strategies. Bioinformatics aims to derive new understanding from large datasets, and an important practical aspect of achieving this is the development of software that can display these complex datasets in a clear and accessible way to allow experimental scientists to interrogate them. To effectively integrate disparate datasets from different "omic" technologies with literature and text searching and sources of patient information to assist target identification and validation is an important objective in bioinformatics currently [104, 105].

9.1 Target Structure

Often useful insights into the role of a target in cell biology can be gained from the sequence of the gene and its mRNA transcripts along with studies of the protein three-dimensional structure. From these it is possible to assign most genes to known structural classes such as GPCRs or kinases and recognise structural domains such as those for DNA binding or kinase active sites. These allow broad inferences to be made about function, types of interaction with other proteins and likely post-translational modification. However, these need experimental confirmation. An example of the value of this information is Aurora-2 kinase where structural bioinformatics was used to identify other kinases with high homology. The crystal structure of one of these (1CDK) was used as a template to generate a 3D homology model from which active site inhibitors were successfully predicted and kinase dead constructs designed for functional validation studies [106].

The existences of differential splicing patterns and isoforms can reveal the complexity of the target biology and also highlight the importance of clearly defining the molecular form(s) believed to be linked to disease and that should be used for further target validation. Scrutiny of the gene sequence beyond the coding region can give pointers to the regulation of the target and how this relates to cell responses, e.g. involvement in inflammatory responses through presence of NFκB transcription factor binding sites in the promoter. Bioinformatic analysis of the DNA sequence of the promoters of genes undergoing changes in expression in Alzheimer's disease for consensus transcription factor binding sites has identified potential common underlying regulatory mechanisms in the disease [107]. Also the 5′ UTR of mRNA can contain regulatory sites such as binding sites for microRNAs with assigned functions (although currently it is hard to predict miR target genes with confidence) [108].

9.2 Expression

An obvious prerequisite to studying the dependence of a cell response or phenotype on the target is to demonstrate the presence of the target in both the key cell in disease and the cell system to be used in vitro for functional studies.

mRNA levels are widely used as a measure of gene expression and taken as a surrogate for actual protein levels largely because of the ease of the analysis. However, this may not always be accurate as post-transcriptional control of translation occurs by many mechanisms, e.g. suppression by microRNAs. Therefore inferences about the target based on mRNA levels require confirmation at the protein level. mRNAs extracted from cells and tissues are mainly measured using gene chips, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), branched DNA probe detection or Northern blots, but these technologies tend to be used in complementary ways.

Gene chip microarrays, of which the Affymetrix system (http://www.affymetrix.com/) is the most widely used version [109], are a sensitive technology for comparing expression of all genes in the genome and how they changes between samples. Affymetrix chips carry multiple hybridisation probes for each gene plus many common splice variants. Chips are also available for microRNAs and for the genome of several animal species such as mouse, rat and rhesus macaque. Although a routine technique, experience and best practice are needed to obtain reliable results that are comparable between experiments and different laboratories ([110] and references therein). Variability in the quality of sample preparation and standardisation of expression microarray experiments and their statistical analysis in the literature has raised concern and resulted in wide acceptance of the MIAME guidelines for reporting of results [110]. Bioinformatic searches of public domain or proprietary databases of gene chip experiments can provide a broader picture of target expression in the body and initiate thinking about possible safety liabilities. Also selective expression in the cell of interest suggests a specific role in that cell.

qRT-PCR is a complementary technique suited to subsequent detailed investigation of a single gene across a range of samples rather than comparison of expression of all genes in the transcriptome. qRT-PCR is more sensitive (e.g. many GPCR mRNAs are expressed below the detection level of gene chips), has a very wide dynamic range and is capable of high throughput including 384-well formats. mRNA is transcribed into cDNA then amplified exponentially using PCR with the accumulation of a fluorescent product that is measured in real time over the range in which product reflects the amount of input cDNA. Good quality results again require careful standardisation and adherence to best practice, and the minimum requirements for reporting qRT-PCR experiments have been proposed also [111]. The TaqMan technology is a widely used example (http://www.lifetechnologies.com/). The preparation of suitable standards for absolute quantitation is difficult, and the most widely used approach is relative quantitation where the expression is described as a ratio relative to a normalising RNA selected for its consistency of expression across all the experimental samples. However

there are no universal normalisation genes, and the best choice needs to be investigated for each study [112, 113]. It is important to note that relative quantitation only allows comparison of the expression level of the same gene across different samples and not between different genes in a sample. The sensitivity of the qRT-PCR methodology means it can be applied, if great care is taken, to mRNA isolated by laser capture of single cells from tissue sections [114]. Methods are available for microRNAs also [115].

There is growing use of the branched DNA methodology which uses a hybridisation probe capable of high signal amplification and can achieve very high sensitivity and dynamic range [116] and can be applied to extracted mRNA or in situ (Panomics: http://www.panomics.com/).

For protein determinations in cell extracts, one- and two-dimensional gel electrophoresis separation and Western blotting using a validated antibody are widely used and can give semi-quantitative results. Enzyme-linked immunosorbent assays (ELISA) employing a capture antibody coated onto a plate and a different antibody linked to an enzyme for detection are frequently the gold standard for protein quantitation. However the advantages of analysing a range of protein components in a single sample in parallel (although with slightly higher incidence of interfering factors) have resulted in various multiplexing technologies [117] such as Luminex (https://www.luminexcorp.com/) where multiple ELISAs are performed with each capture antibody loaded onto one of a 100 different beads distinguishable by flow cytometry. Arraying capture antibodies onto chips or plates to create analytical protein microarrays [118] such as the commercial Meso Scale system (http://www.mesoscale.com/) is becoming widely used for cytokines and signalling pathways. Integral membrane proteins are often difficult to study by the above methods. However measurement of expression on the surface of isolated cells using fluorescent antibody detection by immunocytochemistry (ICC) or flow cytometry is a valuable technique that can explore both the level of protein expression and heterogeneity within a population.

Frequently the availability of suitable antibody reagents can limit which techniques can be used as a given antibody may only be sensitive and specific for a single technique such as Western blotting or ELISA. Raising a suitable antibody de novo can have variable success and needs forward planning. The availability of purified protein or cloned cells expressing an integral membrane protein are essential tools in validating assays to determine protein expression.

Proteomics approaches are used to profile the protein repertoire of cells, tissues or biofluids and post-translational modifications. Detection and quantitation involves fragmentation of proteins to peptides and analysis by various mass spectrometry techniques with or without prior separation techniques [119, 120]. However the relatively low sample throughput, difficulties in detecting low abundance proteins and the instrumentation and data handling demands tend to make this a specialised approach.

The presence of a target protein may also be inferred from its activity providing a suitably specific method is available such as pharmacological profiling using

known agonists and antagonists or, in the case of an enzyme, specific substrates and inhibitors.

These techniques for mRNA and protein determination described for isolated cells can contribute evidence linking the target to cell responses. However when they are applied to homogenates of tissues, they lose the possibility for topological resolution. Therefore in situ hybridisation (ISH) with antisense probes to mRNA and immunohistochemistry (IHC) with specific antibodies on frozen or paraffin-embedded sections of patient tissue can be an important source of evidence as questions can be asked about whether target distribution is co-localised with the appropriate cell and also pathology. An illustration of this is the use of IHC in COPD samples in conjunction with functional studies in mouse smoking models to reveal a previously underappreciated role of IL1α-positive haematopoietic cells in smoke-induced neutrophilic inflammation [121]. ISH can have particular value when identifying cells synthesising a secreted protein. ISH and IHC are at best only semi-quantitative although branched DNA techniques for mRNA may now offer a way forward for tissue slices and whole cells.

If the patient samples are well curated and have associated health and treatment records, then it is possible to stratify the samples according to such criteria as observed pathology, disease activity and stage which could contribute to patient stratification for trials. IHC in patient samples using molecular markers for disease processes such as fibrosis or hypertrophy can also provide fundamental disease understanding and create new hypotheses. Building such a tissue collection curated by a qualified histopathologist can require substantial time and investment but is a highly valuable target validation asset.

9.3 Signal Transduction

Understanding where the target fits into the known intracellular signalling networks can add further valuable evidence about function, for example, involvement in cell division or the inflammatory response. Also if the target is found to lie in a signalling pathway that contains other targets known to be involved in the regulation of the cell response central to the hypothesis, then this can also add confidence.

Over the last two decades, the assay techniques to analyse the different types of signalling events such as intracellular second messenger release, phosphorylation, translocation and protein-protein interaction have become well established. Initially these were applied to cell populations after lysis. However high-content analysis (HCA or high-content screening or HCS) is an approach being used increasingly that combines whole cell imaging with multiparameter readouts sometimes in live cells [122]. This allows single-cell and whole population analyses. Readouts can correlate multiple changes in cell response, organelle morphology and changes such as increased target expression, activation or localisation. Specialised image acquisition instrumentation and analysis software such as ArrayScan (http://www.

thermoscientific.com/en/home.html) and Definiens (http://www.definiens.com/) are central to these techniques. Reporter assays are also a widely used technique to show activation of complete pathways through switching on expression of an indicator transgene such as luciferase that is under the control of a pathway-dependent promoter [123]. A recent development is functional protein microarrays where large numbers of purified proteins are arrayed on a chip and cell lysates applied to study global signalling changes in post-translational processes such as kinase phosphorylation or protein-protein interactions [118].

Profiling of genes for epigenetic markers of activation or repression that involve chemical modification of DNA or its associated histones [124] is becoming a very important area for understanding disease cell biology and identifying targets that may be important in disease-related cell phenotypes.

The discovery of approximately 700 genes whose microRNA products control cell responses through pleiotropic effects on the expression of dozens of functionally related genes is an important area in understanding the regulation of cell phenotypes (as well as representing a new group of therapeutic targets) [125]. Further large groups of small RNA regulators of gene expression and large naturally occurring antisense molecule have been described recently the roles of which in disease are being explored [126].

In the past intracellular signalling studies focused on relatively few linear canonical pathways such as the MAP kinase cascade. However, now signalling processes are being understood more as complex interaction networks that are flexible and vary depending on cell type, stimulus and context. Mining the vast literature and knowledge databases for reported connections between gene products, signalling events and cell phenotypes increasingly benefits from computational biology approaches such as the widely used Ingenuity Pathways Analysis tool (IPA; Ingenuity Systems http://www.ingenuity.com/) that allows integration of multiple "omics" datasets and literature reports. This allows novel interaction networks to be proposed that places the target in an overall signalling context and creates a hypothesis for how it relates to cellular functions. However experimental verification is still needed. An example of this is the use of expression microarray data and IPA to generate an hypothesis about signalling networks and genes causing resistance to erlotinib in NSCLC cell lines that were then validated experimentally [127].

9.4 Functional Validation

9.4.1 Experimental Test Systems In Vitro

Careful definition of the hypothesis in terms of the cell type, cell stimulus and cell response is important in order to develop appropriate experimental cell systems. Despite the abundance of cell systems described in the literature for most organs and diseases, in practice, it is frequently necessary to adapt a cell system or develop one de novo to fit the stimulus and response in the hypothesis or to improve its

relevance to disease by replacing cell lines with human primary cells, for example. This can be a lengthy process and time needs to be allowed in project planning.

Unless the cell response is constitutive, the choice of cell stimulus needs careful consideration. The cell in disease is likely to be exposed to a complex mixture of stimuli that can synergise or one stimulus can prime a cell for activation by another. The intracellular signalling for each stimulus can be different so a target may be wrongly ruled out (or in) if the stimulus is incorrect or if a powerful non-physiological stimulus such as calcium ionophores and, or phorbol ester is used. Conditioned media from cultured cells and biological fluids obtained from disease such as COPD sputum can be a useful approach. It is also good to set the key cell response described in the hypothesis in a broader context by measuring other changes in response to the stimulus by the use, multiplex assays and high-content analysis.

As discussed earlier there are good arguments to support the use of relevant primary cells ideally from patients and culturing them in environments that more closely mimic conditions in vivo. Cell culture on extracellular matrices, cocultures, 3D and organotypic cultures, explants and tissue slices are increasingly being used to deliver more relevant information about cells, their interactions with their environment and their involvement in disease processes. The examples below are just a small number of successes in this area.

In oncology there is a growing understanding of the importance of the interplay between tumour cells and stromal cells [128], much of which has come from coculture experiments. An example relevant to bone metastasis in prostate cancer is the release of bone anabolic and catabolic mediators by tumour cell, osteocyte cocultures [129]. When compared to 2D cultures, the growth of cancer cells in 3D cultures with extracellular matrix has been shown to better reflect an invasive phenotype in prostate cancer cells and strongly influence their sensitivity to drugs such as PI3 kinase inhibitors [130] and also more accurately reflect the action of Her2-targeting reagents in Her-2 amplified breast cancer lines [131].

In respiratory disease, respiratory epithelial cell isolates from donors show a remarkable ability to form an organised striated epithelium in air-liquid interface cultures that closely resemble the transcriptome and morphological features of the original airways [132]. In allergic disease such as asthma cocultures of antigen-presenting cells, lymphocytes and T regulatory cells are central to the study the immune response. Airways in precision-cut lung slices are able to demonstrate bronchodilation and desensitisation to β2-adrenergic agonists [133, 134]. Recently a microfluidic device ("lung-on-a-chip model") has been created that reconstitutes the alveolar-capillary interface between human pulmonary epithelial cells and endothelial cells and subjects them to air and fluid flow and cyclic mechanical strain. Early results suggest that this simulated breathing is important for recreating lung responses seen in vivo [135].

For target identification and validation studies in COPD, asthma and OA at AstraZeneca Charnwood, a wide range of primary cell systems were developed. In COPD a number of cell types were isolated from each sample of lung resection material from emphysematous patients and from noninvolved tissue from lung cancer surgery (see Fig. 3) [136, 137]. Respiratory epithelial cells could be used as air-liquid interface cultures in target biology studies, and an example of

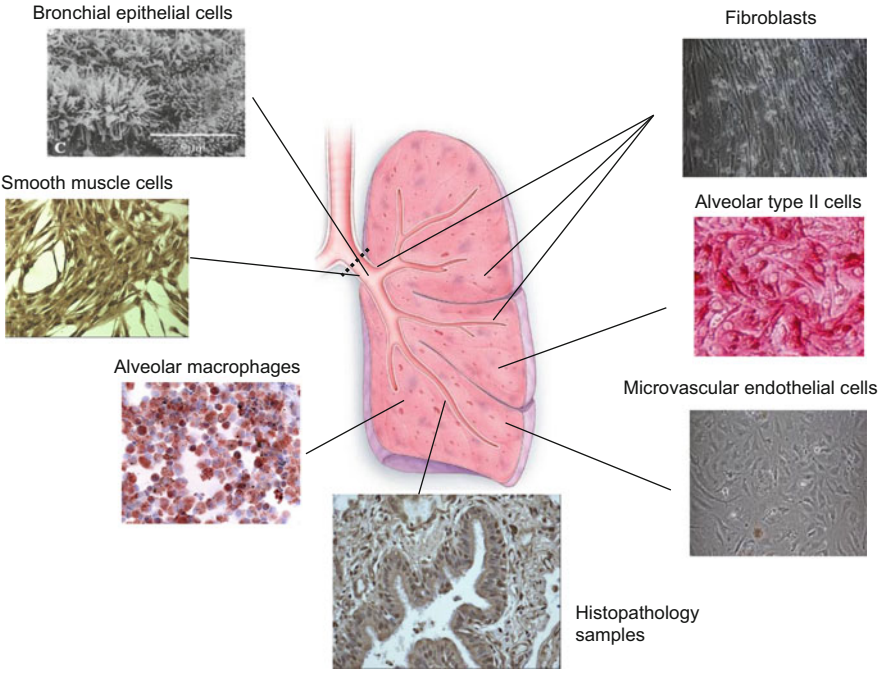

Fig. 3 Uses of human donor lung samples. Human donor lung samples from surgical procedures can provide material for multiple uses in target validation. Cells of different types once isolated can be cryopreserved for later use and are functional. Reproduced from [136] by kind permission of Springer, Holland

the use of alveolar macrophages from such sources was to explore the potential value of targeting p38 MAP kinase in patients whose cells showed a high and low sensitivity to steroids [138]. Such cells can also be used as a gold standard for exploring the utility of more abundant cell systems, e.g. comparison of alveolar macrophages with the properties of monocyte-derived macrophages [139]. Muscle wasting is a symptom found in many COPD patients as well as other conditions and new methods were developed to isolate sufficient satellite cells from post-mortem muscle suitable for high-throughput use [140]. Cartilage explants from OA joint replacement surgery can constitutively release extracellular matrix degradation epitopes, mediators and metaloproteinases and can be further stimulated to do so. We were able to standardise responses in such explants [141] in 96-well formats and similarly in isolated OA chondrocytes. Explants have also been used by others for target validation studies such as for adiponectin and its receptors [142].

Stem cell technology is a potentially valuable approach for target validation and personalised medicine in the future particularly for tissues such as the CNS where primary cells are not easily obtained. Human embryonic stem cells (ESC), adult stem cells (ASC) and induced pluripotent stem cell (iPSC) are able to differentiate under appropriate conditions into various cell types and substructures of different

organs [143]. An early experiment with iPSC derived from a diabetic donor's skin has already been used to culture pancreatic cells showing disease phenotypes [144]. However few well-characterised systems exist as yet (e.g. differentiation of stem cells to neurones tends to result in a mixture of types). This will continue to be a very active area aided greatly by the search for regenerative therapies. A further recent development that could have important applications in target validation is the rotating wall vessel bioreactor which is capable of creating a regular supply of advanced, engineered tissues [145].

With these developments comes an increased range of choices about which cell(s) to use and in what culture system. Again having a well-defined hypothesis for the mechanism by which a target will impact on disease can be useful for focusing on the right choice. It is important to not assume that a more organotypic or multicellular system will automatically be more relevant to disease but instead to set up criteria for validating this based on comparisons with data from patients. The areas of comparison can be the biology of the target (activation, expression, up- and downstream signalling), level of the similarity of the transcriptome and types and strength of cell responses including where possible benchmarking against known drugs.

The methods for modulating target function are described in Sect. 9.4.4. However it is important to recognise that in more complex cocultures and organotypic systems using primary cells, the challenges of delivery of macromolecular reagents such as siRNA and transgene constructs increase markedly. Therefore small molecules can often be the method of choice.

9.4.2 Practical Considerations in the Use of Human Donor Samples

To access human donor material for target validation means overcoming a number of organisational and technical challenges. Several commercial sources of human donor material do exist. However arranging access to human tissue through hospitals or medical research institutes by collaboration and establishing a working relationship with surgeons and researchers with an interest in the study is often a good way to ensure a regular supply of good quality samples. The types and amounts of samples available will depend on the disease and clinical practice. In the case OA, regular samples of disease cartilage and underlying bone are available due to joint replacement therapy, but in RA these are rare, but synovial fluid can be obtained. Of primary importance is that the experimenter specifies and ensures compliance with the protocols for how sample are to be handled and shipped. Although human donor material for functional studies is inherently more variable than cultured cell lines and human material has frequently to undergo lengthy procedures to isolate the required cell type, good experimental reproducibility and low failure rates can be achieved with multiple donors over many months. Regularity of supply can be an issue, and it is a precaution to have more than one source. Establishing such supply can be lengthy as it involves gaining permission from the local ethics committees. Finally there is a need to archive and track human samples in line with prevailing biobank regulations. Inevitably the costs and effort involved in routinely utilising human donor material can be high.

9.4.3 Experimental Systems In Vivo

Very few animal models if any mimic the full spectrum of mechanisms and pathologies of the human disease. Therefore it is necessary to identify models that best recapitulate the specific pathology important to the hypothesis and confirm as far as possible that the mechanisms underlying it parallel those that occur in human disease. The extent to which responses in animal models can translate to improvements in clinical trial end points and patient symptoms depends on the disease with past experience in the clinic showing that greater success is likely in areas such as blood chemistry, lung function tests and inflammation than in areas such as pain.

To gain the best evidence to support an animal model as fit for purpose, it is important to investigate, firstly, how closely the target and its biology compare in the model and man (including structure, cell and tissue distribution, signalling and function in the key cell type for the hypothesis). This may need some supporting studies such as functional validation in appropriate animal cells. Secondly, it is necessary to evaluate the degree of concordance of the pathology in the model with that in human disease and the mechanisms giving rise to it as similar pathologies may arise through different mechanisms [146]. Transcriptomics and histopathology supported by immunohistochemistry of the target and key disease process markers can provide useful points of comparison between the animal model and man as can any therapies active in man that are also active in the animal model species. Finally, for animal models intended to predict an improvement in a clinical trial, end point then is necessary to recognise the wide differences in success rates between diseases as mentioned above. Taking all this characterisation data together, it is important to then assess the overall level of risk and decide how much emphasis to place on the animal model alongside other lines of target validation information rather than making efficacy in an animal model an automatic tollgate for project progression.

An illustration of the value of comparison at the level of histopathology is the generation of bone metastases using the prostate cancer cell line PC3 and the $ARCaP_M$ clone. Both can establish bone metastases following inter-cardial injection. However $ARCaP_M$ is able to establish bone metastases with dominant osteoblastic lesions and some osteoclastic lesions that are histologically similar to human disease, whilst PC3 cells generate largely osteoclastic metastases. Therefore the $ARCaP_M$ system is likely to be more relevant for targets involved in the formation of the metastasis itself (though other criteria for suitability should be applied as described above) [147, 148].

Whilst in compound screening turnaround time and throughput are important, for validation of high priority targets this is not such a consideration. Some of the animal models most able to represent chronic human disease could be long time course models such as the chronic ovalbumin challenge model of asthma [149].

Use of GEMMS in oncology allows the particular mutational background found in patient subsets to be copied in mice. Results from this approach are widely considered to be a great step forward as regards predictivity of drug effects over traditional xenografts due to the tumour arising spontaneously in the correct context of stromal cells and other micro-environmental factors as well as providing more

relevant pharmacokinetic properties for dose to man determination [150, 151]. GEMMs offer an important way forward for patient stratification and personalised medicine approaches for drug testing and potentially for creating hypotheses about patient stratification biomarkers that can then be examined to see if they translate into patients with similar genomic changes. However GEMMS currently are less suitable for studies of metastasis formation. It is usual to use tissue-specific and inducible expression systems for the mutated oncogenes or tumour suppressor genes in GEMMs to achieve good results. However there are still differences from human tumours in certain respects such as the degree of epigenetic modification [152]. A successful application of the GEMMS approach outside cancer is the introduction of the human Apo E transgenes into mice rendering the mouse susceptible to atherosclerotic plaque formation on a high fat diet. This model has been used extensively in atherosclerosis research for drug testing and identifying the Apo E isoforms most important in this and Alzheimer's disease [153]. The growing understanding of the importance of genetic susceptibility factors in diseases should result in wider application of the GEMMS strategy.

Finally, there is an advantage if readouts and biomarkers used in animal models are identical to those to be used in clinical trials as this can reduce possible sources of poor translation from preclinical studies to man. Whole-body imaging by magnetic resonance, PET and CT scans, histology of biopsies and circulating biomarkers can often be used in this way. Novel types of imaging are an important and rapidly expanding area of research [154, 155]. For example, amongst the methods that can be used in COPD in humans and animal models are standard MRI to measure lung oedema (a feature of emphysema), whilst MRI of inhaled hyperpolarised ^{3}He allows real-time imaging of gas flow. Lung vascular perfusion in different regions of the lung can be imaged by magnetic labelling of blood [156].

Clearly it can take a major investment of effort to develop and build a good case that an animal efficacy model mimics aspects of human disease. There is a good rationale for doing this precompetitively in collaborations and consortia to achieve wider and more rapid validation of the model and to share cost.

9.4.4 Techniques for Modulating Target Activity

Modulation of target activity can be achieved by a wide range of techniques including small molecules, aptamers, antibodies and naturally occurring ligands. At the RNA level, antisense and RNA interference can be used and at the DNA level mutations and transgenic constructs. Whilst the lead small molecules from a drug discovery project may be active across species, frequently a parallel probe for use in the animal model needs to be developed, and this is nearly always the case for antibodies and gene silencing reagents. With gene silencing reagents and transgenes, the ability to deliver in vitro and in vivo can be an important hurdle requiring specialised delivery systems.

In using these tools and techniques, it is essential to undertake studies to determine the efficacy and selectivity of the probe and generate supporting

evidence that target modulation in vitro or in vivo has been achieved for the duration of the experiment. Additional supporting studies in the target validation experiment should aim to reveal any evidence that indicates the mode of action is not through an effect on the target. Understanding the potential for false positives and negatives and artefacts for each technology is very important in achieving this as will be described for each.

Synthetic Small Molecules

Despite recent advances in gene silencing, small molecules are still key tools for target validation (though only for the 20% of the genome considered druggable). For relatively novel targets there may be no publications or patents describing small molecule probes. However, a chemical genomic approach using screening of small molecule libraries designed around known pharmacophores of the druggable class has been used with good success by us and others [157]. Fragment libraries and virtual screening have also been used. The medicinal chemistry effort to develop a suitable small molecule probe for studies in vivo is considerably greater as sufficient potency, selectivity and metabolic stability must be built in.

Single compound, single high concentration studies in vitro and in vivo as often reported do not provide sufficient evidence for target involvement in the phenotype and are often misleading due to off-target activities. The potency and efficacy of a probe need to be quantified initially in a test system that is dependent on the target activity such as a receptor cloned into a non-expressing cell background with an immediate downstream signalling readout of activity, e.g. receptor autophosphorylation. The comparison of the pharmacology characterised in the test system with that obtained by dose response studies in the target validation experiment in vitro or in vivo is important in increasing or decreasing confidence in the correct mode of action. It is very important to monitor the pharmacodynamics in vitro and in vivo to confirm an adequate effect of the probe over the time course of the target validation study and provide evidence that the mode of action is as expected, e.g. by using the immediate downstream signalling event used in the test system. Confidence in selectivity should be increased in vitro or in vivo by the sound pharmacology practice of ensuring that at least two structurally unrelated probes give the same phenotypic effect. A functional validation approach with a different mechanism such as RNA interference may also be useful to "phenocopy" the compound effect. Testing for selectivity against closely related targets is useful and is particularly important in the case of kinase inhibitors.

Aptamers

Single-stranded oligonucleotides (20–60 bases long) that assume specific conformations depending on their sequence can act as tight binding, selective modulators of target function [158]. They can be isolated in a few days from high complexity

libraries by repeated rounds of selection of high-affinity binders followed by RT-PCR amplification (the SELEX process) and further optimised by mutation and chemical modified to enhance stability in vitro and in vivo [158]. Significant amounts of pure target protein are needed, and it is considerably more difficult to apply to targets on cells.

Naturally Occurring Ligands

Knowledge of the target biology and naturally occurring agonists, antagonists and substrates can provide a valuable source of validation probes. The size and molecular nature of these ligands are extremely diverse including small molecules and soluble and membrane-bound proteins. Natural ligands and extracellular domains of receptors can often be engineered to generate useful tools such as cytokine sponges and the immunoglobulin fusion protein CTLA4-Ig. Natural substrates can often be a starting point for the design of inhibitors.

Antibodies

Antibodies that inhibit (or in rare cases activate) can be highly potent and selective tools for exploring the function of mainly extracellular targets such as cytokines and cell surface receptors although approaches to try to extend their use for intracellular targets by use of novel delivery systems or expression from transgenes to generate intrabodies are being explored [159]. The success rate in generating neutralising antibodies by standard monoclonal approaches can be low, and druggable target classes such as class A GPCRs and proteases can prove challenging [160]. There are many techniques used to isolate therapeutic antibodies that may be applicable to generating target validation tools if available to the researcher such as screening in vitro ScFv libraries of the human naïve antibody repertoire and additional techniques to increase chances of isolating antibodies against GPCRs. However from this it is clear that it can be a significant investment in time and effort to try to generate a functional antibody de novo without any guarantee of success. Therefore it is particularly valuable if a functional antibody exists elsewhere or can be cloned from a known sequence.

Gene Silencing

A major leap forward in target validation capability came with the development of tools able to exploit the endogenous RNA interference pathway in eukaryotic organisms [161, 162]. For the first time affordable, off the shelf, tools are available to probe the function of all genes. Short (approximately 23 nucleotide) double-stranded RNA molecules when introduced into the cell are able to trigger specific degradation of target mRNAs with the complementary sequence (unlike longer

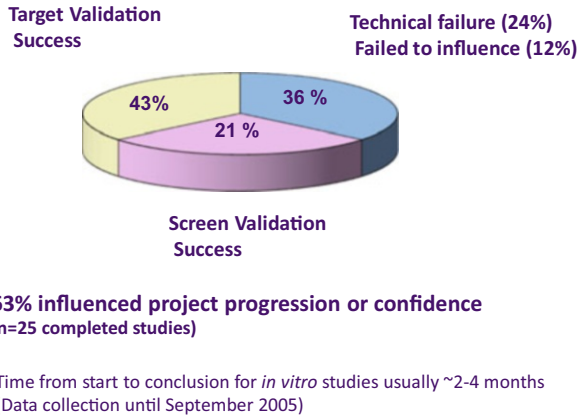

Fig. 4 siRNA impact on milestone transitions in AZ until 2005. Examples of impact (or lack of it) of siRNA in preclinical target progression were elicited by questionnaire from across all the AstraZeneca research areas (all studies were in vitro)

dsRNAs that cause a host defence response). Multiple siRNAs can be predicted for each gene using design algorithms and are commercially available singly or in pools against a single gene and as libraries against sets of genes or the whole genome. Constructs have been generated that can express short hairpin RNAs in the cell and are processed to active siRNAs by the same pathway used for microRNA maturation [162]. Expression of shRNA can be under the control of constitutive or inducible promoters (such as the tetracycline on/off system) and with or without integration into the host genome (e.g. after lentiviral or adenoviral delivery, respectively). shRNA approaches are particularly suitable for experiments longer than one week.

A survey in AstraZeneca after the first 3 years of use of siRNA showed that in 65% of projects where it was employed in vitro, it provided influential data for milestone transitions either through validation of screens or targets (see Fig. 4).

However siRNA is not suitable in all situations, e.g. validating agonist approaches, and can give false positive and negative results under some circumstances. RNAi frequently does not achieve protein knockdowns equivalent to small molecule inhibitions (often due to delivery limitations) increasing the chance of inconclusive or false negative results. Also many proteins have multiple functions and play a structural role as part of a complex. Therefore the effect of siRNA may result from disrupting these complexes and only be predictive of the effect of a gene silencing drug and not a small molecule. Therefore it is important in further validation that a pharmacological probe with similar mode of action to the intended drug is used.

An siRNA can generate off-target effects by mimicking microRNA action if its short seed sequence matches a site in the 3′UTR of the off-target gene leading to translational blockade or mRNA degradation [163]. Whilst siRNAs with the same seed sequence as known microRNAs can be excluded during design, this is still a substantial source of off-target effects as it is hard to predict if the seed sequence of a given siRNA will act as a microRNA. An additional adverse effect of RNAi can be perturbation of endogenous microRNA levels through saturation of the microRNA processing pathway [163].

Some siRNA or shRNA sequences have been shown to generate strong interferon response through activation of toll-like receptors 3, 7 and 8 and other pattern recognition receptors such as RIG-1 leading to cytokine release and cell toxicity. There have been many reports of efficacy in vivo using siRNA synthesised from naturally occurring oligonucleotides. However, this is not recommended as native siRNA has a plasma half-life of around a minute and is rapidly excreted through the kidney and its efficacy has been shown in a number of instances, including oncology models, to be mediated through TLR3-dependent cytokine release [164, 165]. The interferon response and to a degree the microRNA effects of siRNAs (but not shRNA) can be reduced by chemical modifications [163]. Chemical modification particularly on the 2' hydroxyl of the ribose in certain positions has been shown to improve half-life to many hours and strongly reduce toll receptor activation, e.g. alternating on both strands [166].

For validating gene silencing reagents and confirming their mode of action, methods must be available for measuring mRNA and protein knockdown in vitro and in vivo (the latter requiring a sensitive antibody for blotting which can be an issue). If the target is not expressed uniquely in the cell of interest in vivo but is widely distributed, then there must be means by which the cell can be isolated as adjacent tissues may not have received sufficient reagent due to variability of delivery to different sites. Testing for absence of toxicity and interferon responses and use of the 5′-RACE assay to demonstrate (qualitatively) the appropriate mRNA cleavage products are valuable methods for adding confidence that the observed effect is through an RNAi mechanism in vivo [167].

Even more so than for small molecules, an essential quality control measure in siRNA studies is to show siRNAs with different sequences are able to generate the same phenotypic effect and that multiple negative control sequences are without effect. Dose response curves of target knockdown and effect on phenotype are important rather than single concentration studies as these can reveal anomalous behaviour of some sequences.

A key issue is that siRNA and sequences encoding shRNA are not able to penetrate cells efficiently without delivery systems. These are usually based on lipid or nanoparticle transfection agents or conjugation to molecules to achieve targeting or improved uptake [168]. Viral vectors are widely used for shRNA [169]. Cells in vitro can vary extensively in their ease of transfection with some, e.g. T and B cells, being particularly challenging. These delivery systems are frequently the source of cytotoxicity or cell stress that can compromise the readout and require validation as being efficient and having low or negligible effects in their own right. In vitro, combined gene silencing tools and delivery reagents should not impact expression of related or housekeeping genes and should show minimal (less than 10%) toxic effects measured by sensitive measures such as nuclear morphology changes, mitochondrial function and vital dye exclusion rather than LDH release or cell number.

Effective delivery to tissue sites in vivo is still limited. Areas of success are effective liposomal preparations able to achieve efficacy in the liver at sub-microgram/kg levels and lipoplexes able to deliver to lung vasculature [170, 171].

The larger pore size and less well-formed angiogenic vessels in inflammation and tumours allow increased local concentration of siRNA delivered in lipids or other particulates, and many systems claim efficacy in tumour models. Using targeting mechanisms such as antibodies or receptor-mediated uptake has been widely described. The transferrin nanoparticle from Calando has shown target knockdown and efficacy in melanomas in the clinic [172]. Many tissues such as the CNS remain a challenge. MicroRNA mimics are also double-stranded RNAs and require similar delivery systems.

Because of its availability, higher potency and ease of design, siRNA has tended to gain wider use than the earlier gene silencing technology of antisense that utilises single-stranded RNA/DNA hybrid "gapmers" to recruit RNAse H to cleave the complementary sequence in mRNA [173]. However this technology has continued to advance with new chemical modifications to the nucleotide sugars, e.g. locked nucleic acids (LNAs) allowing improved potency and shorter gapmers with significantly greater ability to enter some cells in vitro without a delivery system [174]. An antisense molecule can be designed that is specific to a given target and does not suffer the microRNA off-target effects of siRNA. LNAs and other latest chemistries are able to achieve good circulating levels and half-lives and enter cells to some degree in vivo also without delivery systems [175]. Good potency (low g/kg) can be achieved for tissues that are exposed to high circulating concentrations such as liver and kidney cortex though higher doses are needed for various tumours and tissues. Therefore, delivery systems are likely to still be needed to achieve efficacy and avoid toxicities in many situations (microRNA antagonists are also single stranded and tend to use similar nucleotide chemistries). Antisense may again become more widely used and can be a confirmatory approach for siRNA.

Mutational Analysis

Over-expression of the target protein, genetic knockouts, and the expression of mutated versions with changed activity are widely used approaches to exploring target function in vitro and in vivo. Constitutively active mutations that render the target active independent of cell regulatory control and dominant negative mutants that inactivate the target and compete with the endogenous protein for signal transduction partners are widely used strategies. In certain target classes there are established strategies for creating such mutants, e.g. dominant negative kinases and constitutively active low molecular weight G proteins. Such constructs can be stably integrated and expressed from the chromosome via nonviral or viral vector delivery or remain extrachromosomal (e.g. by adenovirus or adeno-associated viral delivery). The later allows truly isogenic comparisons of mutant and controls as it avoids random gene disruption although it has a limited duration. A significant hurdle can still be efficient transfection particularly in some primary cell types. If transfection efficiency is only moderate, it is often necessary to select using some co-expressed marker. This may exclude use in certain primary cell systems if they are short lived or nondividing.

Although the value of these mutational approaches is well established, there are risks of artefacts as over-expression of a protein (often by an order of magnitude or more) can lead to non-physiological interactions in the cell. Also dominant negatives can monopolise signal transduction elements upstream as well as downstream of the target, e.g. by acting as a substrate for an upstream kinase. If these upstream elements signal into other pathways, then these too will be suppressed even though the target does not lie on them. As with siRNA a genetic knockout may mediate an effect through destabilising a protein complex. The need for confirmation of the results from these mutational strategies using orthogonal approaches such as a small molecule is clear.

Inducible systems in vitro and in vivo, e.g. transgenes under the control of the tetracycline promoter or knockouts by gene excision using an inducible Cre recombinase systems, allow improved experimental control and also overcome the risk of adaptation by the organism during early development. Induction under the control of a growing number of tissue-specific promoter is increasingly being used to more precisely test the hypothesis about the role of the target in disease. A good example of use of all these techniques is the demonstration in mouse of the importance of NFκB activation in the epithelium as a key step in neutrophilic recruitment to the lung in inflammation and the importance of the kinase IKKβ, and not IKKα, in NFκB activation in primary human lung epithelial cells which was then confirmed using selective inhibitor [176–179].

The newly developed CRISR/Cas9 system for gene editing is a powerful and efficient way of creating double allelic knockouts or targeted mutagenesis of the endogenous gene in cell lines or animals without the need to integrate a transgene [180]. It can also be used to edit multiple genes in parallel. The system involves transient expression in a cell of a prokaryotic Cas9 nuclease and a single guide RNA (sgRNA) that contains the sequences homologous to the target site and the sequence required for Cas9 action. This can give rise to double strand breaks and mutations that cause gene knockout or, alternatively by inclusion of an oligonucleotide repair template, can direct specific gene editing. Modified procedures using a mutated Cas9 nickase have been developed to reduce off-target effects [180].

9.5 Human Genetics

Investigating the association between sequence variations in the target gene and a disease phenotype in patients can provide a valuable source of supporting data for the overall hypothesis as it is about the only approach that directly links the target with actual patient symptoms. Sites of heterozygosity occur approximately every 300 base pairs in the genome, and the information from the International HapMap Consortium [92] has provided a powerful tool for these studies. Hybridisation probes can be used in assays to investigate whether SNPs in any candidate gene are associated with disease using suitable case and control cohorts. Positive results

are informative, but lack of an association does not rule out the target as the functional consequences of the SNPs are usually unknown.

Genetics can also provide evidence to support the component hypotheses as shown by the rare inherited deficiency of cathepsin C (dipeptidyl peptidase 1) which results in the complete absence of active neutrophil elastase in the azurophilic granule confirming DPP1 as the nonredundant upstream processing enzyme for the activation of neutrophil elastase [181].

10 Predicting Safety Liabilities Due to the Target

It is a target validation question to ask what the normal physiological role of the target is and what will be the consequences of modulating its activity. Therefore it is important that target validation studies are designed to also allow potential safety liabilities to be flagged.

Profiling the target's involvement in signalling pathways of known function and mRNA expression pattern can give an early indication of how widely expressed the target is and in what cellular activities it may play a role. For protein expression it is useful to explore this in normal tissue sections (tissue arrays often providing a convenient approach). Functional validation experiments in vitro and in vivo should be monitored for adverse effects and signs of safety liabilities (even though in target validation studies in vivo dosing levels are chosen for pharmacological efficacy rather than to define a therapeutic margin).

Data from knockout mice are often used to flag safety liabilities. However care is needed as target expression in a KO mouse is completely removed which is usually more than is achieved or intended with a medicine, and there are the caveats already described for knockouts including the possibility of adaptation during early mouse development. Human genetics may provide insight into the extent of safety liabilities. In the case of the rare DPP1 deficiency (and hence lack of mature neutrophil elastase), there is an occurrence of prepubescent gum disease [181].

Predictive toxicology screens in vitro for the liver, the most common site of adverse effects, are achieving considerable predictivity and sensitivity through use of multiparametric early toxicity readouts such as mitochondrial function and membrane integrity in primary cell systems [182], coculture with Kupffer cells, precision-cut slices and organotypic cultures [183, 184]. This again demonstrates the relevance of primary cells and more complex cell systems as discussed for studies of efficacy. The consequences of modulating target activity on normal liver physiology if expressed there can be investigated in such systems at an early stage in the project using small molecule or other reagents. Predictive toxicology systems in vitro for other tissues are making progress including the use of stem cells, e.g. for the heart muscle and kidney [185, 186].

Overall these lines of evidence can build a hypothesis of the role of a target in normal physiology and highlight potential safety liabilities. It is important that target validation teams have involvement or input from safety assessment expertise

to assess these risks and define future risk mitigation strategies. However, this early preclinical data is often not enough to provide a clear stop decision, but this information can be used to guide further safety studies during the project.

11 Biomarker Selection

The search for new and robust biomarkers has been one of the most active areas of medical research in recent years. A biomarker has been defined by the National Institute of Health as a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses", and there are a number of different types of biomarkers that can contribute to drug discovery and patient care. Definitions are discussed in Wagner [187]. Before treatment is initiated, biomarkers can indicate the likely presence of disease, whilst other biomarkers are useful for predicting important aspects of disease progression such as speed, severity or probability of the occurrence of pathologies such as metastatic disease. Patient stratification or personalised medicine biomarkers aim to dissect the underlying heterogeneity of the disease and stratify patients into groups according to the disease mechanism involved in order to identify potential responders to a therapy aimed at that mechanism (see Sect. 12.2). Biomarkers used during clinical trial have the purpose of (a) demonstrating that the dosing regime has had the predicted effect on target activity, termed target engagement or proof of mechanism (PoM) biomarkers; (b) demonstrating an effect on aspects of the disease (disease-related biomarkers); and (c) being sensitive and early indicators of adverse events (safety biomarkers). Often it is found in these different applications that two or more biomarkers are needed to achieve the necessary level of predictive confidence as exemplified in OA [188].

PoM biomarkers are an essential part of clinical trials as being able to demonstrate that the pharmacokinetic, pharmacodynamic relationship predicted from preclinical studies translates to an adequate level of target modulation is clearly fundamental to the correct interpretation of efficacy data. Measurement of target activity itself or immediate downstream signalling events such as target protein auto-phosphorylation in the case of a receptor tyrosine kinase are frequently used as PoM biomarkers [187].

It is important to deploy biomarkers as exploratory end points to confirm or disprove in the clinic the sub-hypotheses about the role of the target in cell responses and pathologies described in Sect. 7. The information from such biomarkers adds to understanding of disease processes and can assist future target selection. If the cell response or pathology is extensively impacted by the treatment but no efficacy is seen against the primary end point, then further efforts with targets intended to work by the same mechanism are likely to be fruitless. Urinary desmosine levels are increased in COPD and considered to be an indicator of tissue degradation. If inhibition of a lung metalloproteinase did not result in a reduction in desmosine, then it would call into question the target or approach to blocking tissue

degradation as discussed by Vogelmeier et al. [189] for neutrophil elastase (although, in reality, there are usually several possible explanations for not meeting the clinical trial end points).

The development of biomarkers for use in proof of mechanism studies and as exploratory biomarkers is being assisted by the technology developments such as described in Sect. 9 for the analysis of cell signalling events ex vivo, cell responses and circulating mediators and for imaging in vivo. Subsequent to identifying a candidate biomarker is the development of a robust, sensitive, well-characterised and suitable method for its measurement in the clinic (biomarker validation). Aspects of practicality and cost also have to be taken into account when developing diagnostic biomarkers or companion diagnostics for personalised medicine approaches in commonly occurring diseases.

12 Clinical Trial Design

As was shown in Sect. 3.7, clinical trial design can be a cause of attrition. Over the last 10 years, much has been done to try to improve decision-making about progression through Phases 1, 2 and 3. This has included more effective and extensive use of biomarkers, end points and improved patient selection. A consequence of these trends is a growing complexity and cost of clinical trials. Therefore there is a balance to be struck between the number of trials (and hence hypotheses tested) and the complexity and amount of information gathered in each.

Within pharmaceutical companies there has been recognition of the importance of greater integration into the clinical trial design and biomarker selection of preclinical understanding of the molecular and cellular aspects of the target biology and the pharmacology of the candidate drug. Knowledge sharing across preclinical and clinical disciplines and over a period of years is often challenging, but the organisation of drug development projects and their decision-making processes are starting to support this in many cases.

12.1 Choice of Primary and Secondary End Points

The primary end point selected to demonstrate efficacy in a Phase 2 and 3 trial needs to be both appropriate to the claims made for the drug and also be accepted by regulatory authorities on the basis of extensive experience in the clinic. These criteria can be conflicting for first-in-class treatments working by novel mechanisms in diseases such as COPD where the choice of current end point is limited [190]. However there is much effort going into to identifying new end points for COPD [190]. To encourage innovation and successful clinical trial design, bodies such as the FDA are developing sets of guidelines and advice for diseases such as COPD describing recognised end points and strategies for novel drug mechanisms

and encouraging early dialogue with them if a novel end point strategy is proposed. It is very valuable, particularly in Phase 2, if an approved surrogate end point exists as this can be used to give an early indication of efficacy and to support rapid progression. The development of biomarkers as new surrogate end points is receiving much attention, but gaining acceptance for new ones will take time.

A choice of suitably complementary primary and secondary end points can ensure the most value is derived from clinical trials. FEV1 is a quantitative primary end point and can also be used as a surrogate end point. However it is recognised that changes in FEV1 cannot reflect all aspects of COPD. Quality of life health questionnaires have been developed in many diseases including COPD, and some have become recognised as suitable end points. However the risk of subjectivity exists, and the pros and cons of different health questionnaires are argued, e.g. between the St George's Respiratory Questionnaire (SGRQ) and others in COPD [191]. The choice of FEV1 as an objective physiological primary end point and the SGRQ as a secondary end point which, although more subjective, can provide information on the extent to which patient needs have been met is a complementary choice that is often used [189].

12.2 Identifying Responders, Patient Stratification and Personalised Medicine

Intense effort is going on in most disease areas to identify potentially useful patient stratification factors distinguishing patient subsets on the basis of disease mechanism. The importance of such molecular diagnoses was illustrated in Sect. 3.7 for EGFR inhibitors. As would be expected oncology is the lead area in this field given the growing number of detected genomic changes in cancer and the ease by which they can be screened. An early success in improved therapy is the use of KRAS as a stratification biomarker for choice of treatment in metastatic colorectal cancer which has played an important part in tripling of patient median survival time [192]. Huge effort is going into further stratification of cancer patients, development of companion diagnostic assays and identification of effective targeted drugs and combinations. Molecular diagnosis of the mechanism by which resistance to cancer treatments arises is a further area of stratification that will improve trial success and future treatment. The ultimate goal of personalised medicine is to tailor the treatment to the full mutational background of the individual. Approaches to this include culture of primary tumour cells from individual patients using 3D techniques such as described in Sect. 9.4.1 and testing drug combinations to achieve the best outcome [193, 194]. Successes are not limited to cancer. In asthma the importance of selecting allergic versus neutrophilic severe asthmatic patient for Xolair treatment has been mentioned earlier, and recently variants of the β2-adrenergic receptor have been identified as influencing the sensitivity of some asthmatics to β2-agonists [195].

Many other methodologies that are able to measure different changes associated with disease are being investigated in the very active search for stratification and personalised medicine biomarkers including epigenetics, transcriptomics, cellular assays and histopathological analysis of biopsies and imaging techniques [196]. A number of these have been highlighted earlier in the chapter. In COPD there has been a recent proposal for stratifying patients according to a matrix of symptoms and disease severity [197].

However it is necessary to apply appropriate study design to clearly demonstrate the improved predictive power of these stratification biomarkers and companion diagnostics and therapies which may not be a trivial matter [198].

Population and ethnic differences is another key factor being considered in personalised medicine. These can be based on genetic or other differences. For example, in many emerging economies smoking is on the increase compared to Western Europe, so there are likely to be more early-stage COPD patients with consequences for mechanisms and treatment approaches.

13 Effective Management of Target Selection and Progression

13.1 Managing Target Selection

A clear conclusion from 10 years of work in the Respiratory and Inflammation Research Area of AstraZeneca is that the strategy and organisation of target selection and validation can have a profound impact on the productivity of the process. Around 2000 we explored a number of genomic projects for target selection and encountered the challenges inherent in this approach described in Sect. 3.4.2. In order to establish a target selection mechanism that was both more efficient and able to respond flexibly to changing needs of the portfolio and integrate the full range of validation approaches, we created cross-discipline project teams able to drive target selection. Each team had expertise and input from molecular biology, bioinformatics, disease cell biology, immunohistochemistry, in vivo pharmacology, clinical sciences and medicinal chemistry.

The operating model was that each project team would focus on a single TPP and its associated pathologies and cell responses and would establish relevant experimental systems to perform target selection and validation. There was a strong emphasis on developing assays in vitro using regular supplies of patient samples for probing target function with ligands, small molecules and RNAi, etc. and on immunohistochemistry in well-curated disease tissue collections. Other technologies including small expression genomic studies were used as appropriate to contribute complementary data rather than be the central driver for target selection. Each team would use whatever technologies or approaches appeared most productive to find targets according to their assessment of the area and richness of

opportunities from the literature and conference reports. Target ideas were strictly prioritised, and typically 2–3 targets per team were worked on in parallel for a set period of time (usually around 6 months) to a predefined validation strategy. For each target this usually involved substantial in-house experimental work prioritised according to an assessment of the key risks of failure of the target hypothesis. For a target to enter the pipeline, it had to be reviewed successfully against a set of target selection criteria (see Sect. 13.2).

Over a 4-year period, this proved a very efficient mechanism able to deliver one new target into the pipeline per 1.2 FTEs (including effort spent on candidate targets that did not achieve pipeline entry criteria). On average 60% of targets were deselected for various reasons before pipeline entry due to the work of the project teams which resulted in bringing attrition forward before lead generation commenced thus improving the average quality of targets in the pipeline and reducing waste in high-throughput screening and medicinal chemistry effort.

13.2 Importance of Target Selection and Progression Criteria

Defining the criteria for when a target is considered adequately validated to enter the hit identification process and also to move through later drug discovery milestones is central to managing target progression. The way in which validation criteria can make a strongly positive impact on pipeline attrition is again illustrated by experience in respiratory and inflammation at AstraZeneca Charnwood (see Table 2). Here the underlying philosophy was that targets should be examined against a set of criteria on pipeline entry and criteria of increasing stringency at subsequent milestones so that target confidence should be enhanced in step with increasing investment in lead generation.

Prior to March 2003 the pipeline was largely populated with legacy targets selected for various reasons including literature evidence and target class approaches, whilst after this time new targets had to be subjected to a set of more rigorous and standardised validation criteria that frequently necessitated in-house work to provide an adequately robust validation package. A 3–4-fold reduction in the percentage of projects closing for target validation reasons was achieved at each of the two drug discovery stages between target selection and lead optimisation phase. Also pre-2003 much effort was expended prior to project closure on trying to reposition these legacy targets in other disease indications to find an application for the medicinal chemical assets that had been developed. Other metrics of the cost involved showed that the resource expended upfront on target validation was easily justified by the saving on later project attrition before lead optimisation phase. Also in a situation where hit identification capacity is constant, this improved pipeline performance would be expected to result in more projects reaching candidate drug phase which generates more clinical trials or more choice.

Table 2 Value of target validation criteria in reducing attrition in the early preclinical pipeline (AstraZeneca Respiratory and Inflammation Discovery Research)

	Hit identification phase		Lead identification phase	
Period	No. of closure events	Due to lack of efficacy data	No. of closure events	Due to lack of efficacy data
01/01/2000 to 01/03/2003	20	40%	12	33%
01/03/2003 to 13/10/2007	41	12% (inc. 5% due to target toxicity)	25	8% (inc. 4% due to target toxicity)

Project terminations across all AstraZeneca Respiratory and Inflammation Research disease areas were assessed to identify where lack of sufficient supporting efficacy data was the main reason for closure. After 01/03/2003 projects are mainly those that had passed review against more stringent standard target selection criteria for pipeline entry (see Sect. 13.2)

In practice the criteria used by different pharmaceutical organisations vary widely. How these are chosen tends in part to be a practical decision dependent on factors specific to the organisation such as what is the capacity to run lead generation activities, how full is the pipeline at different stages, how much risk of later attrition is tolerable and how important it is to succeed in a given area. Another factor governing the actual criteria is the nature of the disease indication; for example, how much functional validation can be done in vitro. Given that more information and validation tools will arise during the project, criteria for pipeline entry often need to be a suitable compromise. If they are too stringent, then flow of targets may be too low; if they are too weak, then too many will suffer attrition later.

The following are target entry guidelines similar to those used to bring about the improved pipeline performance post 2003 shown in Table 2. It is clear from the following discussion that the decision to start small molecule or biological lead generation on a given target should involve input from multiple disciplines:

Clear hypothesis for efficacy. This needs to explain how modulation of the target activity will result in efficacy to meet the TPP through causative steps such as outlined in Sect. 7 and must be adequately detailed to allow accurate testing of the hypothesis and sub-hypotheses.

Full assessment of public domain and internal data on the target. As a resource, a structured bioinformatics and data mining package should be assembled and maintained describing known genomic variants and isoforms, expression analysis from available databases, signal transduction information and relevant literature on function.

Evidence for target expression at the protein level in disease in the correct cell and tissue (with expression of any relevant ligands or receptors). In addition to mRNA and protein analysis in cell extracts, high-resolution expression analysis in disease tissue (by immunohistochemistry or in situ hybridisation) in well-characterised patient samples is preferable as this can more precisely relate the target expression to the cell types and pathology consistent with the hypothesis.

Functional validation in disease-relevant biological test systems. For most targets and diseases, the minimum requirement for pipeline entry is for this to be shown

in a disease-relevant cell system in vitro. Wherever possible this should employ primary human cell samples. If the TPP requires the target to provide improved efficacy on top of existing therapies, then the test system needs to be configured to show this. Routinely these data would be generated in-house (with any public domain data supporting confidence). An important spin-off of this investment in-house is that the assay systems are ready to be integrated into the screening cascade as required. Also the selectivity, potency and mode of action of functional probes (RNAi, compounds, etc.) must be confirmed by the methods described in Sect. 9.4.4 and an adequate number of independent probes or orthogonal methodologies used to ensure confidence. Additional in-house or public domain data obtained in vivo and human genetic disease association data would clearly provide additional strength. Risk of redundancy regards mechanism or target should have been assessed.

A feasible plan for further validation. To achieve the level of confidence needed for later milestone transitions and ultimately to enter the clinic, a feasible further validation plan is essential. Without this a target may look increasingly weak as the project progresses. A risk assessment should be made to identify key experiments to strengthen the target and mitigate areas of high risk.

Clinical trial feasibility. There is a requirement for a top level input from clinical sciences on the probable shape, feasibility and likely strength of Phase 2 and 3 studies. This ensures early input and engagement from the clinical and development organisation.

Identification of potential safety issues. A review by those with safety assessment expertise of what is known about the target biology including its expression pattern in the body is required. Whilst this rarely results in a stop decision, potential risks will be identified and tests can be incorporated into the project progression plan.

Feasibility of the screening cascade for drug discovery. A screening cascade should be defined based on assays tested in-house or ones for which there is a very low risk of failure. The molecular form of the target used for screening needs to be selected. Choice of SNP variants can influence the structure activity relationship (SAR).

It is important to recognise that target validation considerations are only one of the areas to assess when deciding on whether a target should enter the pipeline. Others are druggability (Sect. 13.3) and assessment of competition. Freedom to operate needs to be assessed as the impact on the project of the patent situation regards the target and drug discovery methodology is important particularly in the area of monoclonal antibody discovery.

The package of target validation data supporting pipeline entry is generally only a starting point, and it is important that this package is strengthened during the early phases of drug discovery driven by the target validation plan which may need updating in the light of new results. This risk mitigation plan is often specific to the target. However in general terms the target validation picture should be expected to develop preclinically in the following areas:

Expression. More extensive immunohistochemistry against tissue sections from patients to relate the target to disease processes using markers and to disease stage and patient segments.

Functional validation. With the development of better validation probes through the lead identification and optimisation process, then it is likely that data will have been obtained in additional or more disease representative functional validation systems in vitro and in vivo. If an efficacy model in vivo is used, an assessment supporting its relevance will have been made as described in Sect. 9.4.3. Other potential mechanisms involving the target that might contribute to efficacy or risks of redundancy could have been investigated.

Human genetics. A genetic association study to identify linkage to disease occurrence or specific disease traits will have been done.

Biomarker and personalised medicine strategy. This will have been defined preclinically.

Mechanism-related safety issues. Progress will have been made to mitigate risk through predictive toxicology or other means as described in Sect. 10.

It is important to try to define clear go/no go decision criteria if possible and adhere to them despite the caveats and uncertainties that sometimes surround functional validation test systems and probes.

Use of external experts to provide an independent and 360-degree assessment of the reason to believe in a project through means such as scientific advisory boards often happens late on when it tends to be too late to reasonably question the selection of the target. Therefore there is a strong case for including independent experts with a range of opinion in a review of target validity earlier before selecting a candidate drug.

13.3 Druggability in Target Selection

Current experience in small molecule drug discovery is that it is easier to identify hits and leads for certain classes of target than for others and is therefore a dominant factor in assessing the risk associated with a target. In general GPCRs, kinases, nuclear hormone receptors, enzymes, transporters and ion channels are considered to be the most druggable, whereas protein-protein interactions are considered to be difficult or undruggable [199]. However it is important to look in more depth as some subsets of protein-protein interactions clearly are amenable to blockade, e.g. chemokine and chemokine receptor interactions, whilst some subsets of ion channels and even class A GPCRs have proved difficult. Also it's important to recognise that being able to find initial hits may not translate into developing a safe new chemical entity with good drug-like properties. Kinase active site inhibitors can be identified easily, but developing adequate selectivity can hinder drug development. Also it can prove difficult to develop drug-like active site inhibitors for lipases due to the hydrophobicity of the substrate-binding pocket.

Therefore when evaluating the feasibility of lead generation, it is important to consider the following additional questions that can help assess risk:

- Are small molecule inhibitors already reported in publications and patents and have good pharmaceutical properties? Is it known that others have been unsuccessful in identifying small molecule hits?
- If no significant small molecule inhibitors are known, then how close in sequence and structure is the target to others in its class where inhibitors are known?
- Has the target been crystallised, or can computation chemistry develop a homology model based on the crystal structure of a related target to assist drug discovery by co-crystallisation or virtual screening?

A danger in such analyses is that we become limited by past experience and do not discover ways to develop small molecules against novel target classes. The discovery in our labs of a new class of immunomodulators acting through a previously undrugged monocarboxylate transporter subclass is an example [94]. Therefore it is appropriate for druggability to be a strong factor that influences the target selection strategy overall but should not totally exclude other targets which present with a strong validation package.

Today it is usual for large pharmaceutical companies to have access to additional therapeutic platforms such as monoclonal antibodies, fragment antibodies, therapeutic proteins and oligonucleotides. This greatly increases the opportunity to develop medicines for the most validated targets so reducing the risk of project attrition. Monoclonal antibodies and therapeutic proteins which are directed against extracellular or cell surface targets increase the number of available therapeutic targets to around 6,000 [200]. Antisense and siRNA therapeutics to any therapeutic target inside or outside the cell can be designed readily. Also oligonucleotide therapeutics can achieve total selectivity between very similar structures, e.g. kinases. However, there are still major delivery issues to be overcome. The further development of oligonucleotide delivery technologies should be of high importance to the pharmaceutical sector as many of the most validated targets for different diseases lie in the 75% of the genome that is still considered undruggable.

13.4 Portfolio Management

Target selection and validation can play an important role in overall portfolio management to meet the strategic needs of the pharmaceutical organisation by focusing effort on TPPs that have been prioritised because of a shift in disease area priority or fluctuation in pipeline occupancy. Company strategies undergo review often resulting in a need to reshape the portfolio rapidly to maintain productivity. The Disease Target Project team model used by AstraZeneca Respiratory and Inflammation Research Area described in Sect. 13.1 proved very flexible and responsive to such changes after a major strategic review resulted in closure of osteoarthritis as a disease area and the parallel development of novel TPPs in the remaining respiratory

and inflammation indications. The project teams were a fully dedicated and experienced resource that could be quickly realigned to new disease biology and transfer experimental skills to new TPPs. New targets were selected and accepted into the pipeline to largely reshape the early portfolio in only a few years.

Monitoring the severity and type of risks associated with individual targets and factoring in the different levels of risk between disease areas described in 3.1 can assist portfolio management to achieve the desired risk level in pipelines for a single disease or across the whole portfolio.

14 Conclusions

At the start of the millennium, prospects for an increase in drug discovery looked bright. The sequencing of the human genome along with a growing understanding of gene expression changes in disease, signalling pathways and cancer-causing oncogenes fuelled an understandable optimism that their would be greater success in the clinic and an increased number of novel medicines each year. The fact that this has not happened despite increased investment has caused much loss of confidence amongst investors and deep self-examination within the sector. Much discussion has centred on the preclinical technologies and strategies used for target selection and validation. However this chapter has sought to show that, whilst this is an important factor, the root causes are broader and the search for solutions needs to be more holistic.

Commercial considerations as well as patient need have driven a trend towards higher risk portfolios focused on first-in-class approaches in chronic and severe diseases. Whilst it is very important to strive to meet these patient needs, proper evaluation and balancing of risk across a drug discovery portfolio is a crucial activity for modern pharmaceutical companies. The pressure to maintain a full development pipeline to provide an optimistic outlook for investors can fuel late attrition and be ultimately counterproductive if pursued at the expense of maintaining rigorous standards of target validation. Increasingly companies are becoming open to in- and out-licensing and co-development deals, and these provide options to use their development infrastructure more flexibly and also to spread and balance risk.

Should a candidate drug achieve registration, there is still a significant risk of attrition in practice due to failure to make a profit and not be taken up by payers. Essential considerations in mitigating this risk are correctly aligning the TPP with well-researched patient need, constructive engagement with payers and those evaluating cost-effectiveness and follow-up “in life” studies where necessary and realistic pricing strategies.

A clear conclusion from many of the failures in the clinic due to lack of efficacy is that previously we have underestimated the complexity of disease processes. However, the drug discovery statistics reflect the historic status of knowledge. In the time between current late phase projects entering the drug discovery pipeline and the present day, there have been major advances in our understanding.

These include basic regulatory mechanisms such as microRNAs and epigenetics, identifying new disease mechanisms and understanding the importance in many diseases of the patient's genomic background as well as describing mechanistically distinct patient groups within a given disease. New potential predictive and personalised medicine biomarkers are being identified at an impressive rate. If even some of these prove robust and useful in clinical practice, then important progress will have been made in selecting the right patient for the right treatment. How far we have progressed on the journey towards being able to adequately understand disease processes at the molecular level so we can accurately predict the outcome of modulating a given target in a given patient in prevalent diseases is still unknown, but it is likely that we are still some way off.

The challenge to understand disease processes better and establish better biomarkers and predictive preclinical models has resulted in a very valuable move towards collaboration, precompetitive research, public private partnerships and open access initiatives and away from the previous industry emphasis on protecting intellectual property rights as the highest priority. The emergence of RNA interference as a widely used target validation workhorse would not have been possible without a more open attitude towards intellectual property. Although it is early days, the move towards full openness in clinical trials data and "big data" initiatives such as searchable electronic health records may yet make further contributions to our understanding of disease, our susceptibility to it and occurrence of comorbidities. These trends towards increased openness and collaboration are likely to accelerate the growth of our understanding of disease and development of useful methodologies with subsequent benefits for drug discovery.

The developments in understanding of disease mechanisms, biomarker validation and patient segmentation are already having a positive impact on clinical trial design. The emergence over recent years of collaborative working between regulators and companies should speed acceptance of biomarkers and new end points. Also the wider use of secondary and exploratory end points should result in better understanding of the effect of medicines in trials and feedback information on disease processes.

Today many strategies for candidate target identification are available. Traditional knowledge-based strategies have been enhanced by the growing public domain information sources and bioinformatic approaches developed to mine them. Powerful functional genomic techniques and methods for genome-wide scans for genetic associations with disease have come to the fore to add to other earlier hypothesis-free "omics" approaches. Drug repurposing seeks to ensure that maximum value is derived from existing drug development efforts. Major investments have been made in systems biology and the next 10 years should reveal how productive it is likely to be in target identification and disease state modelling.

In the past the gap in functional validation technologies and the failure to develop predictive biological test systems in vitro and in vivo have undoubtedly led to many false positive targets entering the clinic. Progress in areas such as RNA interference, primary cells, 3D and organotypic cultures and GEMMs shows how these barriers are being pushed back. However, there are no short cuts to good

quality functional validation data, and the demands in technical skill, time and resources to establish these approaches have to be recognised and made available. Developing such biological test systems and validating (as far as possible) that they properly recapitulate features of human disease is a resource-intensive but essential activity as is the proper characterisation of functional probes and their mode of action. Using human donor material in test systems in vitro requires the setting up of a reliable supply infrastructure and overcoming technical challenges to achieve reproducibility. It is argued here that improved target validation justifies these efforts given that the cost is still small compared to failed Phase 2 and 3 trials. An area that is also showing clearly the benefit of primary, 3D, coculture and organotypic cultures is predictive toxicology for more accurate detection of safety liabilities which is itself a target validation issue if they are the result of the target biology.

The value has been described here of rigorously formulating the hypothesis for efficacy in a series of causative steps or sub-hypotheses for the role of the target in the key cell response, the cell in pathology and the pathology in clinical trial primary end points. This forms a basis for a thorough assessment of risk and development of mitigation plans as part of the target validation strategy.

In target selection and validation at AstraZeneca in Respiratory and Inflammation, a key learning in achieving high productivity and reducing attrition in the early pipeline was the importance of how the process was organised and quality controlled. Organisationally, the most productive model was the creation of dedicated multidisciplinary teams each aligned to a TPP with yearly objectives for successful target delivery against clear target selection criteria. The teams were free to adopt whatever strategies or technologies were most appropriate given the nature and abundance of target opportunities. Two concepts were central to this strategy. Firstly, it was recognised that target validation is a discipline in its own right requiring the development of a group of scientist with in-depth knowledge of the best practice, strengths and weaknesses of key technologies and who were experienced in identifying target validation risk and designing and managing validation strategies. Secondly, the team composition recognised the importance of fully integrating preclinical and clinical expertise.

The insights of network pharmacology about the potential danger of the dominant “One Target, One Disease” paradigm and the value in identifying effective target combinations deserves careful investigation. Developing efficient strategies to identify these novel combinations could be challenging but of high value.

An assumption that is often inherent in target selection and progression is that there will always be a large number of targets for every TPP, and if small molecule hit identification fails against one, then another can be found. However it is quite likely that the number is actually very low when one considers the fraction of all genes likely to be involved in diseases and the percentage that are druggable [200] and taking into account the highly specific nature of many TPPs that may specify, for example, that the target shows additional efficacy on top of existing standards of care. Therefore a target with a good validation status has to be seen as a highly valuable commodity and all efforts made to identify candidate drugs. The gene

silencing modalities of antisense or siRNA have potential for the future as they have high success rates in identifying lead molecules against all targets. However delivery is still a major obstacle for many tissue sites, and cost per patient can be a limitation. Therefore it is to be hoped that the recent decreased investment by the pharmaceutical industry in gene silencing and delivery technologies is reversed in the near future.

Overall, the response to the poor productivity in drug discovery in terms of increased disease research, development of new technologies, new clinical trial approaches and new ways of working has been vigorous and wide ranging. Unfortunately the benefit these developments may deliver will take 10 years to impact on perceived success in the clinic and the industry metrics due to the long drug discovery cycle time. Unfortunately there are strong pressures on industry leadership to deliver rapid improvements. Mergers are one measure to try to improve a company's profitability [201]. But other analyses by the Tufts Centre indicate that drug discovery productivity can suffer [202]. Retrenchment of internal research and closer association with academia in shared facilities to increase innovation, aid translational research and reduce the cost base has becoming a common strategy that is as yet unproven, could be slow to reverse and has led to the loss of capacity and expertise. Undoubtedly the sector has and will continue to go through a period of radical change and organisational upheaval. It is to be hoped that this period of change does not hinder the recent scientific developments described here from delivering their potential. Whilst none of these developments in drug discovery research is likely to be a panacea in its own right, if a number can contribute an incremental improvement such that there is a doubling of the success rate in the clinic from its current level, then it would likely be transformational to the health of the sector. Therefore despite the uncertainties there seems reason to be hopeful that these wide-ranging developments will eventually deliver the desired improved productivity.

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References

1. Paul SM et al (2010) How to improve R&D productivity: the pharmaceutical industry's grand challenge. Nat Rev Drug Discov 9(3):203–214
2. Kaitin KI (2012) Translational research and the evolving landscape for biomedical innovation. J Investig Med 60(7):995–998
3. Pammolli F, Magazzini L, Riccaboni M (2011) The productivity crisis in pharmaceutical R&D. Nat Rev Drug Discov 10(6):428–438
4. Reichert JM (2009) Probabilities of success for antibody therapeutics. mAbs 1(4)387–389

5. Sartori V et al (2011) Value-driven drug development unlocking the value of your pipeline [Online]., http://www.mckinsey.com/Client_Service/Pharmaceuticals_and_Medical_Products/Expertise/Research_and_Development
6. Schulze U, Ringel M (2013) What matters most in commercial success: first-in-class or best-in-class? Nat Rev Drug Discov 12(6):419–420
7. Mastrandrea LD (2010) Inhaled insulin: overview of a novel route of insulin administration. Vasc Health Risk Manag 6:47–58
8. Forst T et al (2009) Time-action profile and patient assessment of inhaled insulin via the Exubera device in comparison with subcutaneously injected insulin aspart via the FlexPen device. Diabetes Tech Therapeut 11(2):87–92
9. Lee EYHP, Muller WJ (2010) Oncogenes and tumor suppressor genes. Cold Spring Harbor Perspect Biol 2(10):a003236. [Online] http://cshperspectives.cshlp.org/content/2/10/a003236.full. Accessed 2 May 2014
10. Perez R et al (2013) A view on EGFR-targeted therapies from the oncogene-addiction perspective. Front Pharmacol 4(April):53. [Online] http://journal.frontiersin.org/Journal/10.3389/fphar.2013.00053/full. Accessed 2 May 2014
11. Lambert GK et al (2013) The background, discovery and clinical development of BCR-ABL inhibitors. Drug Discov Today 18(19–20):992–1000
12. Amit L et al (2013) The impact of bevacizumab (avastin) on survival in metastatic solid tumors – a meta-analysis and systematic review. PLoS One 8(1):e51780. [Online] http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0051780. Accessed 2 May 2014
13. Mitchison TJ (2012) The proliferation rate paradox in antimitotic chemotherapy. Mol Biol Cell 23(1):1–6
14. Frankfurt OS, Krishan A (2003) Apoptosis-based drug screening and detection of selective toxicity to cancer cells. Anticancer Drugs 14(7):555–561
15. Ruiz C, Tolnay M, Bubendorf L (2012) Application of personalized medicine to solid tumors: opportunities and challenges. Swiss Med Wkly 142:w13587. http://www.smw.ch/content/smw-2012-13587/. Accessed 2 May 2014
16. Barker BL, Brightling CE (2013) Phenotyping the heterogeneity of chronic obstructive pulmonary disease. Clin Sci Lond England 124(6):371–387
17. Bernstein PR (1998) Chemistry and structure–activity relationships of leukotriene receptor antagonists. Am J Respir Crit Care Med 157(6 Pt 2):S220–S225; discussion S225–S226, S247–S248
18. Heilker R et al (2009) G-protein-coupled receptor-focused drug discovery using a target class platform approach. Drug Discov Today 14(5–6):231–240
19. Pytliak M et al (2011) Serotonin receptors – from molecular biology to clinical applications. Physiol Res Acad Scientiarum Bohemoslovaca 60(1):15–25
20. Lander ES et al (2001) Initial sequencing and analysis of the human genome. Nature 409(6822):860–921
21. Betz UA, Farquhar R, Ziegelbauer K (2005) Genomics: success or failure to deliver drug targets? Curr Opin Chem Biol 9(4):387–391
22. Hopkins AL (2008) Network pharmacology: the next paradigm in drug discovery. Nat Chem Biol 4(11):682–690
23. Barbaric I, Miller G, Dear TN (2007) Appearances can be deceiving: phenotypes of knockout mice. Brief Funct Genomics Proteomics 6(2):91–103
24. Chan DA, Giaccia AJ (2011) Harnessing synthetic lethal interactions in anticancer drug discovery. Nat Rev Drug Discov 10(5):351–364
25. Kaelin WG (2005) The concept of synthetic lethality in the context of anticancer therapy. Nat Rev Cancer 5(9):689–698
26. Goekoop-Ruiterman YPM et al (2005) Clinical and radiographic outcomes of four different treatment strategies in patients with early rheumatoid arthritis (the Best Study): a randomized, controlled trial. Arthritis Rheum 58(2 Suppl):S126–S135

27. Prinz F, Schlange T, Asadullah K (2011) Believe it or not: how much can we rely on published data on potential drug targets? Nat Rev Drug Discov 10(9):712. [Online] http://www.nature.com/nrd/journal/v10/n9/full/nrd3439-c1.html. Accessed 2 May 2014
28. Austin CP et al (2004) The knockout mouse project. Nat Genet 36(9):921–924
29. Sacca R et al (2010) Genetically engineered mouse models in drug discovery research. Meth Mol Biol (Clifton, N.J.) 602:37–54
30. Joulia-Ekaza D, Cabello G (2007) The myostatin gene: physiology and pharmacological relevance. Curr Opin Pharmacol 7(3):310–315
31. Miles PD et al (2000) Improved insulin-sensitivity in mice heterozygous for PPAR-gamma deficiency. J Clin Investig 105(3):287–292
32. Zambrowicz BP, Sands AT (2003) Knockouts model the 100 best-selling drugs–will they model the next 100? Nat Rev Drug Discov 2(1):38–51
33. Zambrowicz BP, Turner CA, Sands AT (2003) Predicting drug efficacy: knockouts model pipeline drugs of the pharmaceutical industry. Curr Opin Pharmacol 3(5):563–570
34. Cree IA, Glaysher S, Harvey AL (2010) Efficacy of anti-cancer agents in cell lines versus human primary tumour tissue. Curr Opin Pharmacol 10(4):375–379
35. Neve RM et al (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10(6):515–527
36. Stillie R et al (2009) The functional significance behind expressing two IL-8 receptor types on PMN. J Leukocyte Biol 86(3):529–543
37. Bevaart L, Vervoordeldonk MJ, Tak PP (2010) Evaluation of therapeutic targets in animal models of arthritis: how does it relate to rheumatoid arthritis? Arthritis Rheum 62(8): 2192–2205
38. Greek R, Menache A (2013) Systematic reviews of animal models: Methodology versus epistemology. Int J Med Sci 10(3):206–221
39. Berge O-G (2011) Predictive validity of behavioural animal models for chronic pain. Br J Pharmacol 164(4):1195–1206
40. McCarthy MI, Smedley D, Hide W (2003) New methods for finding disease-susceptibility genes: impact and potential. Genome Biol 4(10):119. [Online] http://genomebiology.com/2003/4/10/119. Accessed 2 May 2014
41. Bush WS, Moore JH (2012) Genome-wide association studies. PLoS Computat Biol 8(12): e1002822. [Online] http://www.ploscompbiol.org/article/info%3Adoi%2F10.1371%2Fjournal.pcbi.1002822. Accessed 2 May 2014
42. Benkirane M et al (1997) Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5delta32. J Biol Chem 272(49):30603–30606
43. Rossol M et al (2009) Negative association of the chemokine receptor CCR5 d32 polymorphism with systemic inflammatory response, extra-articular symptoms and joint erosion in rheumatoid arthritis. Arthritis Res Ther 11(3):R91. [Online] http://arthritis-research.com/content/11/3/R91. Accessed 2 May 2014
44. Perry CM (2010) Maraviroc: a review of its use in the management of CCR5-tropic HIV-1 infection. Drugs 70(9):1189–1213
45. Fleishaker DL et al (2012) Maraviroc, a chemokine receptor-5 antagonist, fails to demonstrate efficacy in the treatment of patients with rheumatoid arthritis in a randomized, double-blind placebo-controlled trial. Arthritis Res Ther 14(1):R11. [Online] http://arthritis-research.com/content/14/1/R11. Accessed 2 May 2014
46. Gerlag DM et al (2010) Preclinical and clinical investigation of a CCR5 antagonist, AZD5672, in patients with rheumatoid arthritis receiving methotrexate. Arthritis Rheum 62(11):3154–3160
47. Lindner E et al (2007) Lack of association between the chemokine receptor 5 polymorphism CCR5delta32 in rheumatoid arthritis and juvenile idiopathic arthritis. BMC Med Genet 8:33. [Online] http://www.biomedcentral.com/1471-2350/8/33. Accessed 2 May 2014
48. Woodruff PG (2011) Novel outcomes and end points: biomarkers in chronic obstructive pulmonary disease clinical trials. Proc Am Thorac Soc 8(4):350–355

49. Thatcher N et al (2004) Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). Lancet 366(9496): 1527–1537
50. Lee CK et al (2013) Impact of EGFR inhibitor in non-small cell lung cancer on progression-free and overall survival: a meta-analysis. J Natl Cancer Inst 105(9):595–605
51. Bierma-Zeinstra SMA, Verhagen AP (2011) Osteoarthritis subpopulations and implications for clinical trial design. Arthritis Res Ther 13(2):213. [Online] http://arthritis-research.com/content/13/2/213. Accessed 2 May 2014
52. Knopman DS (2008) Clinical trial design issues in mild to moderate Alzheimer disease. Cognit Behav Neurol 21(4):197–201
53. Guengerich FP (2011) Mechanisms of drug toxicity and relevance to pharmaceutical development. Drug Metabol Pharmacokinet 26(1):3–14
54. Dabu-Bondoc S, Franco S (2008) Risk-benefit perspectives in COX-2 blockade. Curr Drug Saf 3(1):14–23
55. Sewing A et al (2008) Helping science to succeed: improving processes in R&D. Drug Discov Today 13(5–6):227–233
56. Johnstone C, Pairaudeau G, Pettersson JA (2011) Creativity, innovation and lean sigma: a controversial combination? Drug Discov Today 16(1–2):50–57
57. Scottish Medicines Consortium (2011) A guide to quality adjusted life years. [Online] http://www.scottishmedicines.org.uk/About_SMC/Policy_Statements/A_Guide_to_Quality_Adjusted_Life_Years. Accessed 25 Apr 2014
58. Gyrd-Hansen D (2005) Willingness to pay for a QALY: theoretical and methodological issues. PharmacoEconomics 23(5):423–432
59. Bobinac A et al (2013) Valuing QALY gains by applying a societal perspective. Health Econ 22(10):1272–1281
60. Farahani P, Levine M, Goeree R (2006) A comparison between integrating clinical practice setting and randomized controlled trial setting into economic evaluation models of therapeutics. J Eval Clin Pract 12(4):463–470
61. Chiu Y et al (2012) Access to the next wave of biologic therapies (Abatacept and Tocilizumab) for the treatment of rheumatoid arthritis in England and Wales: addressing treatment outside the current NICE guidance. Clin Rheumatol 31(6):1005–1012
62. Humbert M et al (2005) Benefits of omalizumab as add-on therapy in patients with severe persistent asthma who are inadequately controlled despite best available therapy (GINA 2002 step 4 treatment): INNOVATE. Allergy 60(3):309–316
63. Brown R et al (2007) Cost-effectiveness of omalizumab in patients with severe persistent allergic asthma. Allergy 62(2):149–153
64. McKeage K (2013) Omalizumab: a review of its use in patients with severe persistent allergic asthma. Drugs 73(11):1197–1212
65. NICE (2013) TA278 Asthma (severe, persistent, patients aged 6+, adults) – omalizumab (rev TA133, TA201): guidance. http://publications.nice.org.uk/omalizumab-for-treating-severe-persistent-allergic-asthma-review-of-technology-appraisal-guidance-ta278/evidence-and-interpretation#summary-of-appraisal-committees-key-conclusions. Accessed 5 Sept 2013
66. Niedergassel B, Leker J (2009) Open innovation: chances and challenges for the pharmaceutical industry. Future Med Chem 1(7):1197–1200
67. Weigelt J (2009) The case for open-access chemical biology. A strategy for pre-competitive medicinal chemistry to promote drug discovery. EMBO Rep 10(9):941–945
68. Krleza-Jerić K, Lemmens T (2009) 7th Revision of the Declaration of Helsinki: good news for the transparency of clinical trials. Croatian Med J 50(2):105–110

69. Yao L et al (2011) Electronic health records: implications for drug discovery. Drug Discov today 16(13–14):594–599
70. Jensen PB, Jensen LJ, Brunak S (2012) Mining electronic health records: towards better research applications and clinical care. Nat Rev Genet 13(6):395–405
71. Coorevits P et al (2013) Electronic health records: new opportunities for clinical research. J Inter Med 274(6):547–560
72. Denny JC et al (2011) Variants near FOXE1 are associated with hypothyroidism and other thyroid conditions: using electronic medical records for genome- and phenome-wide studies. Am J Hum Genet 89(4):529–542
73. Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8(12):958–969
74. Graff JW et al (2012) Cigarette smoking decreases global microRNA expression in human alveolar macrophages. PLoS One 7(8):e44066. [Online] http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0044066
75. Chung KF, Adcock IM (2008) Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. Eur Respir J 31(6):1334–1356
76. Liu Y et al (2013) Efficacy of anti-interleukin-5 therapy with mepolizumab in patients with asthma: a meta-analysis of randomized placebo-controlled trials. PLoS One 8(3):e59872. [Online] http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0059872. Accessed 2 May 2014
77. Damiano VV et al (1986) Immunolocalization of elastase in human emphysematous lungs. J Clin Investig 78(2):482–493
78. Shao MXG, Nadel JA (2005) Neutrophil elastase induces MUC5AC mucin production in human airway epithelial cells via a cascade involving protein kinase C, reactive oxygen species, and TNF-alpha-converting enzyme. J Immunol (Baltimore, MD) 175(6):4009–4016
79. Vlahos R et al (2012) Glucocorticosteroids differentially regulate MMP-9 and neutrophil elastase in COPD. PLoS One 7(3):e33277. [Online] http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0033277. Accessed 2 May 2014
80. Shalem O et al (2014) Genome-scale CRISPR-Cas9 knockout screening in human cells. Science (New York, N.Y.) 343(6166):84–87
81. Brass AL et al (2008) Identification of host proteins required for HIV infection through a functional genomic screen. Science (New York, N.Y.), 319(5865):921–926
82. Swinney DC, Anthony J (2011) How were new medicines discovered? Nat Rev Drug Discov 10(7):507–519
83. Puri N et al (2008) LNA incorporated siRNAs exhibit lower off-target effects compared to 2′-OMethoxy in cell phenotypic assays and microarray analysis. Nucleic Acids Symp (52): 25–26
84. Whitehurst AW et al (2007) Synthetic lethal screen identification of chemosensitizer loci in cancer cells. Nature 446(7137):815–819
85. Wang Y et al (2010) Critical role for transcriptional repressor Snail2 in transformation by oncogenic RAS in colorectal carcinoma cells. Oncogene 29(33):4658–4670
86. Wurdak H et al (2010) An RNAi screen identifies TRRAP as a regulator of brain tumor-initiating cell differentiation. Cell Stem Cell 6(1):37–47
87. Gumireddy K et al (2009) KLF17 is a negative regulator of epithelial-mesenchymal transition and metastasis in breast cancer. Nat Cell Biol 11(11):1297–1304
88. Echeverri CJ, Perrimon N (2006) High-throughput RNAi screening in cultured cells: a user's guide. Nat Rev Genet 7(5):373–384
89. Bhinder B, Djaballah H (2013) Systematic analysis of RNAi reports identifies dismal commonality at gene-level and reveals an unprecedented enrichment in pooled shRNA screens. Comb Chem High Throughput Screen 16(9):665–681
90. Buehler E, Chen Y-C, Martin S (2012) C911: a bench-level control for sequence specific siRNA off-target effects. PLoS One 7(12):e51942. [Online] http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0051942. Accessed 2 May 2014

91. Buehler E, Khan AA, et al (2012) siRNA off-target effects in genome-wide screens identify signaling pathway members. Sci Rep 2:428. [Online] http://www.nature.com/srep/2012/120529/srep00428/full/srep00428.html. Accessed 2 May 2014
92. Altshuler D et al (2005) A haplotype map of the human genome. Nature 437(7063): 1299–1320
93. Tamari M, Tanaka S, Hirota T (2013) Genome-wide association studies of allergic diseases. Allergol Int 62(1):21–28
94. Murray CM et al (2005) Monocarboxylate transporter MCT1 is a target for immunosuppression. Nat Chem Biol 1(7):371–376
95. Kass DA (2011) Res-erection of Viagra as a heart drug. Circulation Heart Failure 4(1):2–4
96. Jin G, Wong STC (2013) Toward better drug repositioning: prioritizing and integrating existing methods into efficient pipelines. Drug Discov Today. doi:10.1016/j.drudis.2013.11.005. [Online] http://www.sciencedirect.com/science/article/pii/S1359644613003991. Accessed 2 May 2014
97. Turner NC et al (2008) A synthetic lethal siRNA screen identifying genes mediating sensitivity to a PARP inhibitor. EMBO J 27(9):1368–1377
98. Calverley P et al (2003) Conclusion. Lessons from the novel D2 dopamine receptor, beta2-adrenoceptor agonist, Viozan: chronic obstructive pulmonary disease and drug development implications. Respir Med 97(Suppl A):S71–S74
99. Tabernero J et al (2013) First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. Can Discov 3(4):406–417
100. Riethmüller G (2012) Symmetry breaking: bispecific antibodies, the beginnings, and 50 years on. Canc Immun 12:12. [Online] http://cancerimmunity.org/v12p12/. Accessed 2 May 2014
101. Galizzi J-P, Lockhart BP, Bril A (2013) Applying systems biology in drug discovery and development. Drug Metabol Drug Interact 28(2):67–78
102. Hwang W-C, Zhang A, Ramanathan M (2008) Identification of information flow-modulating drug targets: a novel bridging paradigm for drug discovery. Clin Pharmacol Therapeut 84(5): 563–572
103. Diamond SL (2013) Systems biology of coagulation. J Thromb Haemostasis 11(Suppl 1): 224–232
104. Zhou X, Wong STC (2008) Computational systems bioinformatics and bioimaging for pathway analysis and drug screening. Proc IEEE Inst Electr Electron Eng 96(8):1310–1331
105. Yang Y, Adelstein SJ, Kassis AI (2011) Integrated bioinformatics analysis for cancer target identification. Meth Mol Biol (Clifton, N.J.) 719:527–545
106. Vankayalapati H et al (2003) Targeting aurora2 kinase in oncogenesis: a structural bioinformatics approach to target validation and rational drug design. Mol Cancer Therapeut 2(3): 283–294
107. Augustin R et al (2011) Bioinformatics identification of modules of transcription factor binding sites in Alzheimer's disease-related genes by in silico promoter analysis and microarrays. Int J Alzheimers Dis 2011:154325. [Online] http://www.hindawi.com/journals/ijad/2011/154325/citations/. Accessed 2 May 2014
108. Mu W, Zhang W (2012) Bioinformatic resources of microRNA Sequences, gene targets, and genetic variation. Front Genet 3:31. [Online] http://journal.frontiersin.org/Journal/10.3389/fgene.2012.00031/full. Accessed 2 May 2014
109. Auer H, Newsom DL, Kornacker K (2009) Expression profiling using affymetrix GeneChip microarrays. Meth Mol Biol (Clifton, N.J.) 509:35–46
110. Raspe E, Decraene C, Berx G (2012) Gene expression profiling to dissect the complexity of cancer biology: pitfalls and promise. Semin Cancer Biol 22(3):250–260
111. Bustin SA et al (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55(4):611–622
112. Zhai Z, Yao Y, Wang Y (2013) Importance of suitable reference gene selection for quantitative RT-PCR during ATDC5 cells chondrocyte differentiation. PLoS One 8(5):e64786. [Online] http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0064786. Accessed 2 May 2014

113. Hruz T et al (2011) RefGenes: identification of reliable and condition specific reference genes for RT-qPCR data normalization. BMC Genom 12:156. [Online] http://www.biomedcentral.com/1471-2164/12/156. Accessed 2 May 2014
114. Erickson HS et al (2009) Quantitative RT-PCR gene expression analysis of laser microdissected tissue samples. Nat Protocols 4(6):902–922
115. Balcells I, Cirera S, Busk PK (2011) Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers. BMC Biotechnol 11:70. [Online] http://www.biomedcentral.com/1472-6750/11/70. Accessed 2 May 2014
116. Schwartz JR et al (2012) A facile, branched DNA assay to quantitatively measure glucocorticoid receptor auto-regulation in T-cell acute lymphoblastic leukemia. Chin J Cancer 31(8):381–391
117. Leng SX et al (2008) ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. J Gerontol Series A Biol Sci Med Sci 63(8):879–884
118. Zhu H, Cox E, Qian J (2012) Functional protein microarray as molecular decathlete: a versatile player in clinical proteomics. Proteomics Clin Appl 6(11–12):548–562
119. Bruce C et al (2013) Proteomics and the analysis of proteomic data: 2013 overview of current protein-profiling technologies. Curr Protoc Bioinformatics. doi:10.1002/0471250953.bi1321s41
120. Langley SR et al (2013) Proteomics: from single molecules to biological pathways. Cardiovasc Res 97(4):612–622
121. Botelho FM et al (2011) IL-1α/IL-1R1 expression in chronic obstructive pulmonary disease and mechanistic relevance to smoke-induced neutrophilia in mice. PLoS One 6(12):e28457. [Online] http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0028457. Accessed 2 May 2014
122. Zock JM (2009) Applications of high content screening in life science research. Comb Chem High Throughput Screen 12(9):870–876
123. Mayer-Kuckuk P et al (2004) Role of reporter gene imaging in molecular and cellular biology. Biol Chem 385(5):353–361
124. Martin DIK, Cropley JE, Suter CM (2011) Epigenetics in disease: leader or follower? Epigenetics 6(7):843–848
125. Satoh J-I (2012) Molecular network analysis of human microRNA targetome: from cancers to Alzheimer's disease. BioData Mining 5(1):17. [Online] http://www.biodatamining.org/content/5/1/17. Accessed 2 May 2014
126. Martin L, Chang HY (2012) Uncovering the role of genomic "dark matter" in human disease. J Clin Investig 122(5):1589–1595
127. Xu Z-H et al (2013) RAF1-MEK1-ERK/AKT axis may confer NSCLC cell lines resistance to erlotinib. Int J Clin Exp Pathol 6(8):1493–1504
128. Joyce JA, Pollard JW (2009) Microenvironmental regulation of metastasis. Nat Rev Cancer 9(4):239–252
129. Fizazi K et al (2003) Prostate cancer cells-osteoblast interaction shifts expression of growth/survival-related genes in prostate cancer and reduces expression of osteoprotegerin in osteoblasts. Clin Cancer Res 9(7):2587–2597
130. Härmä V et al (2010) A comprehensive panel of three-dimensional models for studies of prostate cancer growth, invasion and drug responses. PLoS One 5(5):e10431. [Online] http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0010431. Accessed 2 May 2014
131. Weigelt B et al (2010) HER2 signaling pathway activation and response of breast cancer cells to HER2-targeting agents is dependent strongly on the 3D microenvironment. Breast Cancer Res Treat 122(1):35–43
132. Pezzulo AA et al (2011) The air-liquid interface and use of primary cell cultures are important to recapitulate the transcriptional profile of in vivo airway epithelia. Am J Physiol Lung Cell Mol Physiol 300(1):L25–L31
133. Cooper PR, Panettieri RA (2008) Steroids completely reverse albuterol-induced beta(2)-adrenergic receptor tolerance in human small airways. J Aller Clin Immunol 122(4):734–740

134. Sanderson MJ (2011) Exploring lung physiology in health and disease with lung slices. Pulmonary Pharmacol Ther 24(5):452–465
135. Huh D et al (2012) A human disease model of drug toxicity-induced pulmonary edema in a lung-on-a-chip microdevice. Sci Translational Med 4(159):159ra147. [Online] http://stm.sciencemag.org/content/4/159/159ra147.long. Accessed 2 May 2014
136. Dougall IG (2011) The use of human tissue in drug discovery. Cell Tissue Bank 12(1):7–8
137. Mackay LS et al (2013) Isolation and characterisation of human pulmonary microvascular endothelial cells from patients with severe emphysema. Respir Res 14:23. [Online] http://respiratory-research.com/content/14/1/23. Accessed 2 May 2014
138. Ratcliffe MJ, Dougall IG (2012) Comparison of the anti-inflammatory effects of Cilomilast, Budesonide and a p38 Mitogen activated protein kinase inhibitor in COPD lung tissue macrophages. BMC Pharmacol Toxicol 13:15. [Online] http://www.biomedcentral.com/2050-6511/13/15. Accessed 2 May 2014
139. Ratcliffe MJ et al (2007) Activation of E-prostanoid4 and E-prostanoid2 receptors inhibits TNF-alpha release from human alveolar macrophages. Eur Respir J 29(5):986–994
140. Scott IC et al (2013) Large-scale isolation of human skeletal muscle satellite cells from post-mortem tissue and development of quantitative assays to evaluate modulators of myogenesis. J Cachexia Sarcopenia Muscle 4(2):157–169
141. Liu YZ, Jackson AP, Cosgrove SD (2009) Contribution of calcium-containing crystals to cartilage degradation and synovial inflammation in osteoarthritis. Osteoarthritis Cartilage 17(10):1333–1340
142. Kang EH et al (2010) Adiponectin is a potential catabolic mediator in osteoarthritis cartilage. Arthritis Res Ther 12(6):R231. [Online] http://arthritis-research.com/content/12/6/R231. Accessed 2 May 2014
143. Bilic J, Izpisua Belmonte JC (2012) Concise review: induced pluripotent stem cells versus embryonic stem cells: close enough or yet too far apart? Stem Cells (Dayton, Ohio) 30(1):33–41
144. Jang J et al (2012) Disease-specific induced pluripotent stem cells: a platform for human disease modeling and drug discovery. Exp Mol Med 44(3):202–213
145. Barrila J et al (2010) Organotypic 3D cell culture models: using the rotating wall vessel to study host-pathogen interactions. Nat Rev Microbiol 8(11):791–801
146. Bolton SJ et al (2009) Characterisation of the proximal airway squamous metaplasia induced by chronic tobacco smoke exposure in spontaneously hypertensive rats. Respir Res 10:118. [Online] http://respiratory-research.com/content/10/1/118. Accessed 2 May 2014
147. Morrissey C et al (2010) The expression of osteoclastogenesis-associated factors and osteoblast response to osteolytic prostate cancer cells. Prostate 70(4):412–424
148. Zhau HY et al (1996) Androgen-repressed phenotype in human prostate cancer. Proc Natl Acad Sci USA 93(26):15152–15157
149. Locke NR et al (2007) Comparison of airway remodeling in acute, subacute, and chronic models of allergic airways disease. Am J Respir Cell Mol Biol 36(5):625–632
150. Combest AJ et al (2012) Genetically engineered cancer models, but not xenografts, faithfully predict anticancer drug exposure in melanoma tumors. Oncologist 17(10):1303–1316
151. Politi K, Pao W (2011) How genetically engineered mouse tumor models provide insights into human cancers. J Clin Oncol 29(16):2273–2281
152. Diede SJ et al (2013) Fundamental differences in promoter CpG island DNA hypermethylation between human cancer and genetically engineered mouse models of cancer. Epigenetic 8(12):1254–1260
153. Hauser PS, Narayanaswami V, Ryan RO (2011) Apolipoprotein E: from lipid transport to neurobiology. Progr Lipid Res 50(1):62–74
154. Olson P et al (2011) Imaging guided trials of the angiogenesis inhibitor sunitinib in mouse models predict efficacy in pancreatic neuroendocrine but not ductal carcinoma. Proc Natl Acad Sci USA 108(49):E1275–E1284

155. Hockings PD et al (2002) Repeated three-dimensional magnetic resonance imaging of atherosclerosis development in innominate arteries of low-density lipoprotein receptor-knockout mice. Circulation 106(13):1716–1721
156. Hopkins SR et al (2007) Advances in magnetic resonance imaging of lung physiology. J Appl Physiol (Bethesda, MD) 102(3):1244–1254
157. Kubinyi H (2006) Chemogenomics in drug discovery. Ernst Schering Res Foundation Workshop (58):1–19
158. Pendergrast PS et al (2005) Nucleic acid aptamers for target validation and therapeutic applications. J Biomol Tech 16(3):224–234
159. Sato M et al (2013) Single domain intrabodies against WASP inhibit TCR-induced immune responses in transgenic mice T cells. Sci Rep 3:3003. [Online] http://www.nature.com/srep/2013/131021/srep03003/full/srep03003.html. Accessed 2 May 2014
160. Hutchings CJ, Koglin M, Marshall FH (2010) Therapeutic antibodies directed at G protein-coupled receptors. mAbs 2(6):594–606
161. Elbashir SM et al (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411(6836):494–498
162. Seyhan AA, Ryan TE (2010) RNAi screening for the discovery of novel modulators of human disease. Curr Pharmaceut Biotechnol 11:735–756
163. Kanasty RL et al (2012) Action and reaction: the biological response to siRNA and its delivery vehicles. Mol Ther 20(3):513–524
164. Kleinman ME et al (2008) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature 452(7187):591–597
165. Bergé M et al (2010) Small interfering RNAs induce target-independent inhibition of tumor growth and vasculature remodeling in a mouse model of hepatocellular carcinoma. Am J Pathol 177(6):3192–3201
166. Robbins M, Judge A, MacLachlan I (2009) siRNA and innate immunity. Oligonucleotides 19(2):89–102
167. Judge AD et al (2009) Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. J Clin Investig 119(3):661–673
168. Lee J-M, Yoon T-J, Cho Y-S (2013) Recent developments in nanoparticle-based siRNA delivery for cancer therapy. BioMed Res Int 2013:782041. [Online] http://www.hindawi.com/journals/bmri/2013/782041/. Accessed 2 May 2014
169. Sliva K, Schnierle BS (2010) Selective gene silencing by viral delivery of short hairpin RNA. Virol J 7:248. [Online] http://www.virologyj.com/content/7/1/248. Accessed 2 May 2014
170. Love KT et al (2010) Lipid-like materials for low-dose, in vivo gene silencing. Proc Natl Acad Sci USA 107(5):1864–1869
171. Aleku M et al (2008) Atu027, a liposomal small interfering RNA formulation targeting protein kinase N3, inhibits cancer progression. Cancer Res 68(23):9788–9798
172. Davis ME et al (2010) Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature 464(7291):1067–1070
173. Prakash TP (2011) An overview of sugar-modified oligonucleotides for antisense therapeutics. Chem Biodiversity 8(9):1616–1641
174. Soifer HS et al (2012) Silencing of gene expression by gymnotic delivery of antisense oligonucleotides. Meth Mol Biol(Clifton, N.J.) 815:333–846
175. Lindholm MW et al (2012) PCSK9 LNA antisense oligonucleotides induce sustained reduction of LDL cholesterol in nonhuman primates. Mol Ther 20(2):376–381
176. Skerrett SJ et al (2004) Respiratory epithelial cells regulate lung inflammation in response to inhaled endotoxin. Am J Physiol Lung Cell Mol Physiol 287(1):L143–L152
177. Cheng D et al (2007) Airway epithelium controls lung inflammation and injury through the NF-kappa B pathway. J Immunol (Baltimore, MD) 178(10):6504–6513
178. Newton R et al (2007) Repression of inflammatory gene expression in human pulmonary epithelial cells by small-molecule IkappaB kinase inhibitors. J Pharmacol Exp Therapeut 321(2):734–742

179. Catley MC et al (2005) Validation of IKK beta as therapeutic target in airway inflammatory disease by adenoviral-mediated delivery of dominant-negative IKK beta to pulmonary epithelial cells. Br J Pharmacol 145(1):114–122
180. Ran FA et al (2013) Genome engineering using the CRISPR-Cas9 system. Nat Protocols 8(11):2281–2308
181. Méthot N et al (2008) In vivo inhibition of serine protease processing requires a high fractional inhibition of cathepsin C. Mol Pharmacol 73(6):1857–1865
182. O'Brien PJ et al (2006) High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. Arch Toxicol 80(9):580–604
183. Sahi J, Grepper S, Smith C (2010) Hepatocytes as a tool in drug metabolism, transport and safety evaluations in drug discovery. Curr Drug Discov Technol 7(3):188–198
184. Catania JM, Pershing AM, Gandolfi AJ (2007) Precision-cut tissue chips as an in vitro toxicology system. Toxicol In Vitro 21(5):956–961. [Online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1986647/. Accessed 2 May 2014
185. Schaaf S et al (2011) Human engineered heart tissue as a versatile tool in basic research and preclinical toxicology. PLoS One 6(10):e26397. [Online] http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0026397. Accessed 2 May 2014
186. Cheng H, Force T (2010) Molecular mechanisms of cardiovascular toxicity of targeted cancer therapeutics. Circ Res 106(1):21–34
187. Wagner JA (2008) Strategic approach to fit-for-purpose biomarkers in drug development. Ann Rev Pharmacol Toxicol 48:631–651
188. Cibere J et al (2009) Association of biomarkers with pre-radiographically defined and radiographically defined knee osteoarthritis in a population-based study. Arthritis Rheum 60(5):1372–1380
189. Vogelmeier C et al (2012) A randomised, placebo-controlled, dose-finding study of AZD9668, an oral inhibitor of neutrophil elastase, in patients with chronic obstructive pulmonary disease treated with tiotropium. COPD 9(2):111–120
190. Martinez FJ, Donohue JF, Rennard SI (2011) The future of chronic obstructive pulmonary disease treatment-difficulties of and barriers to drug development. Lancet 378(9795):1027–1037
191. Puhan MA et al (2007) Relative responsiveness of the Chronic Respiratory Questionnaire. St. Georges Respiratory Questionnaire and four other health-related quality of life instruments for patients with chronic lung disease. Respir Med 101(2):308–316
192. Heinemann V et al (2013) Targeted therapy in metastatic colorectal cancer – an example of personalised medicine in action. Cancer Treat Rev 39(6):592–601
193. Nagourney RA et al (2012) Functional profiling to select chemotherapy in untreated, advanced or metastatic non-small cell lung cancer. Anticanc Res 32(10):4453–4460
194. Golan T et al (2014) Ascites-derived pancreatic ductal adenocarcinoma primary cell cultures as a platform for personalised medicine. Br J Cancer 110(9):2269–2276
195. Portelli M, Sayers I (2012) Genetic basis for personalized medicine in asthma. Expert Rev Respir Med 6(2):223–236
196. European Society of Radiology (2011) Medical imaging in personalised medicine: a white paper of the research committee of the European Society of Radiology (ESR). Insights Imaging 2(6):621–630
197. Agusti A, MacNee W (2013) The COPD control panel: towards personalised medicine in COPD. Thorax 68(7):687–690
198. Dunn G et al (2013) Integrating biomarker information within trials to evaluate treatment mechanisms and efficacy for personalised medicine. Clin Trials (Lond England) 10(5):709–719
199. Gashaw I et al (2011) What makes a good drug target? Drug Discov Today 16(23–24):1037–1043

200. Betz UAK (2005) How many genomics targets can a portfolio afford? Drug Discov Today 10(15):1057–1063
201. Cha M, Lorriman T (2014) Why pharma megamergers work. [online] http://www.mckinsey.com/Client_Service/Pharmaceuticals_and_Medical_Products/Expertise/Research_and_Development. Accessed 25 Apr 2014
202. Getz KA, Kaitin KI (2012) Open innovation: the new face of pharmaceutical research and development. Expert Rev Clin Pharmacol 5(5):481–483

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Optimizing Pharmacokinetic Properties and Attaining Candidate Selection

Keith W. Ward

Abstract The optimization of pharmacokinetic properties of potential new drug molecules is a key component of most drug discovery efforts; failure to achieve this optimization is a major reason for delays and failures in discovery programs. Numerous experimental tools are available to drug discovery scientists with which to evaluate the pharmacokinetic properties of new chemical entities; however, the outcomes from these studies can be complex, and the decision-making paradigm with given experimental outcomes is not always intuitive. This chapter highlights some of the primary risk areas for pharmacokinetics-related attrition, discusses merits and deficiencies of some assays commonly used in a pharmacokinetics lead optimization paradigm, and provides some recommendations for strategies to design an effective pharmacokinetic experimental critical path to provide molecules with optimal developability profiles.

Keywords Bioavailability, Clearance, Pharmacokinetic, Volume

Contents

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Abbreviations and Symbols

A:B	Apical to basolateral directional permeability
AMU	Atomic mass units
B:A	Basolateral to apical directional permeability
cLogP	Calculated log of the octanol:water partition coefficient
CYP450	Cytochrome P450
F	Oral bioavailability
IC50	Concentration producing 50% inhibition
PXR	Pregnane X receptor

1 Introduction

During the discovery of new pharmaceutical agents, the optimization of pharmacokinetic properties plays a central role. The pharmacokinetics of a molecule control its rate of appearance at the site of action, the maximum concentration and extent of exposure at the target site, the rate and means of disappearance of the parent drug molecule from the site of action, and ultimately the elimination of the agent from the body. Therefore, successfully identifying and nominating a candidate compound for progression to development that carries the greatest probability of success is highly dependent on the selection of the molecule with optimal pharmacokinetics, not for the sake of pharmacokinetic properties themselves, but insofar as they support efficacy and safety. Entire libraries and peer-reviewed journals are dedicated to the technical details of the science and art of pharmacokinetic lead optimization and the nuances of the various available assays to explore pharmacokinetic properties, and these will not be recapitulated here. Rather, the purposes of this chapter are to highlight some of the key means by which pharmacokinetics can contribute to attrition risk, describe approaches to minimize the risk of attrition and/or to mitigate the possible downstream consequences of a suboptimal pharmacokinetic profile, and point out potential pharmacokinetic issues and pitfalls that may be encountered during the lead optimization and candidate selection process. Particular attention will be paid to assays which, while often used in pharmacokinetic lead optimization, may not afford the most streamlined process to support research programs.

2 Pharmacokinetics-Related Attrition

The importance of optimizing pharmacokinetic properties as a means to reduce attrition is far from a novel concept. Indeed, as the pharmaceutical industry began to generate increased numbers of new molecules and development programs in the 1980s, it became clear that inappropriate pharmacokinetic properties was one of the key drivers of clinical attrition, being responsible for up to 40% of the failures of development programs in a 1991 survey [1] (Fig. 1). As a result of recognizing this dependence of development success on pharmacokinetic optimization, the industry invested heavily in the late 1980s and 1990s in preclinical pharmacokinetic lead optimization technologies and staff, with the result that it would today be virtually inconceivable for a drug discovery organization to not have access to such preclinical pharmacokinetic data. This investment has been one of the success stories in drug discovery; a more recent survey has suggested that pharmacokinetics-related clinical attrition has decreased to only about 10% of all drug development failure (Fig. 1). Certainly this metric speaks to the value of rigorous consideration of pharmacokinetics during the discovery process. However, it is also important to consider the remaining contributors to attrition; issues with efficacy and safety contribute approximately 25–30% each to the failure of development programs. While some of these failures are almost certainly due to inherent properties of the potential target or chemotype being investigated, it is likely that at least some of this attrition is also pharmacokinetics-related – simplistically, too much or too little active drug reaching the site of action or off-target effect for an inappropriate period of time. Accordingly, further attention to pharmacokinetic issues may well allow continued improvement in overall rates of clinical success.

To address pharmacokinetics-related drug attrition, most organizations engaged in lead optimization place pharmacokinetic assays at key roles along a drug discovery critical path. The simplified generic critical path shown in Fig. 2 is illustrative of a typical route followed by a drug discovery team in moving from lead identification to candidate selection. Embodied in the activities described in this critical path is an acknowledgment that each assay being conducted is designed to eliminate those compounds that are least likely to demonstrate clinical success. With respect to pharmacokinetics, the key attrition risks addressed along this critical path include drug–drug interactions, inappropriate pharmacokinetic properties, and pharmacokinetic contributors to poor efficacy and safety; each of these will be treated in detail below. One of the guiding principles to bear in mind throughout these discussions is that all studies performed in the lead optimization phase should be highly rationale-based. That is, one should not embark upon work in this stage unless there is a clear answer to the question "What will we do with the data?" There is often a great temptation to perform studies, or even to place assays on the critical path, that may be of interest to further characterize a compound, or to explore an interesting pharmacokinetic phenomenon, and it is the role of the effective chemist or DMPK professional to avoid these temptations. Time is the critical determinant in this phase: for a new entity with $1B in annual sales, each day of delay in progressing the ultimate

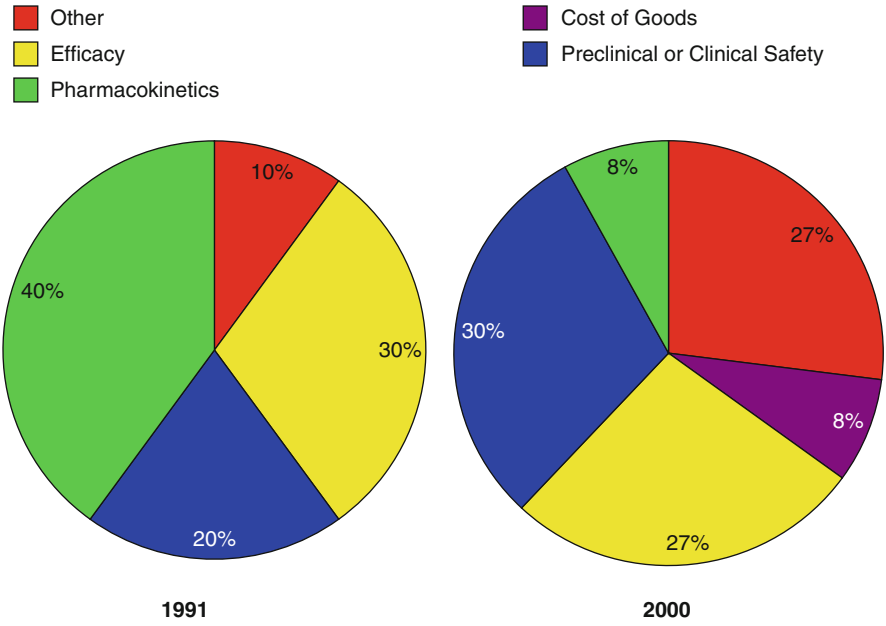

Fig. 1 Increased investment in preclinical pharmacokinetics during lead optimization and candidate selection has resulted in a dramatic decrease in pharmacokinetics-related failure of clinical development programs during the 1990s. Re-depiction of data described by [1]

marketed molecule towards clinical development and launch represents nearly $3MM in lost revenue. Although quality is always important in designing such assays, studies during this phase should be designed with "decision-making quality" – no more, no less. This principle of designing studies for speed and only to answer critical questions should be kept in mind throughout this chapter.

3 Computational Pharmacokinetics

It should be noted that the exemplar critical path displayed in Fig. 2 is highly experimental-based, with no explicit treatment of "in silico" or computational predictive assays. Certainly computational modeling has been effective in chemical design, and to some extent in the prediction of potency of new chemical entities. Progress also has been made with the computational prediction of cytochrome P450 affinity, and in some instances parameters such as protein binding [2]. Given this progress, and the dramatic increases in computational power and predictive algorithms in recent years, many attempts have been made to build global models for the prediction of pharmacokinetic parameters. The concept of performing pharmacokinetic lead optimization in silico is alluring; in theory, success in this area could take a process that may require months or years of iterative experimental time and reduce it to a very short set

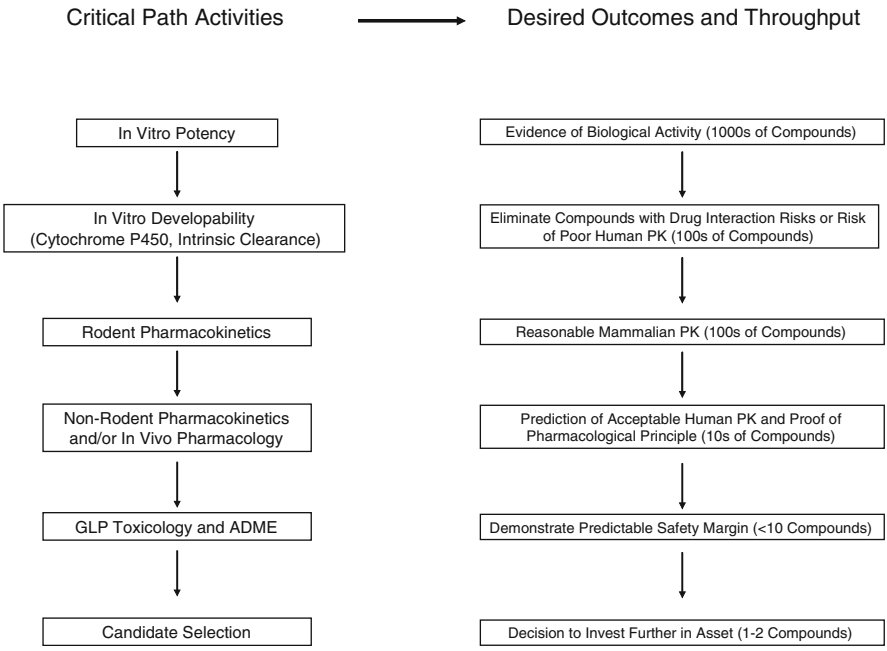

Fig. 2 The *left panel* depicts a typical drug discovery critical path, with higher-throughput or higher failure-rate assays placed early in the screening cascade. On the *right* is shown the intended outcome by stage, as well as the general annual throughput often achieved. Note the key role of DMPK assays in this simplified lead optimization scheme

of confirmatory experiments to validate the model output. Indeed, models with binary output (a pharmacokinetic parameter predicted to be "favorable" or "unfavorable") abound and have enjoyed wide usage in the industry for at least a decade [3, 4]. However, generally speaking, computational models have not been broadly successful with accurate quantitative predictions of pharmacokinetic parameters [5]. Drug metabolism and disposition is a complex process involving multiple interacting biological systems, with a large network of competing reactions, alternative pathways, nonlinear activities, and interindividual variability, and our understanding of these interwoven processes is not sufficient to build global predictive models, particularly for in vivo pharmacokinetics. In some instances, greater success has been encountered with the use of local models, that is, models to predict pharmacokinetic behavior within a single chemical series. However, even in these instances, a fairly large amount of experimental data is required within the series of interest to allow the identification of trends that can be used to build a local model.

More problematic still, for a computational model to be able to predict features in a molecule that will yield improved pharmacokinetic performance, the algorithm must be "trained," ideally with exemplar molecules that have both favorable and unfavorable attributes. Otherwise the software will be unable to accurately report when a molecular change is or is not likely to result in a favorable outcome, since

the model has insufficient experience to know what "favorable" looks like. The catch-22 of this paradigm is that generally speaking, if a lead optimization team has already produced a sufficient number of molecules with favorable pharmacokinetic properties to build a reliable model, the very need for the model is somewhat obviated, in that the team likely has clear empirical structure-performance direction, if not candidate molecules themselves. This general statement is not always applicable, of course; there may be instances where favorable pharmacokinetic properties are coupled with some other unfavorable developability attribute (patent issues, toxicity, drug–drug interactions, etc.), and these molecules could be used to train a computational model. In such instances, local models may be used to prioritize compounds for further study, or even to direct chemistry away from synthesis of a molecule predicted in silico to be suboptimal. However, until the predictive quality of these models is improved, the chemist must be cautious in using such model output in a true decision-making capacity. Otherwise, a team could find itself either eliminating compounds or series via in silico predictions that would in fact have acceptable experimental properties, or spending significant time performing "spot check" experiments to validate the in silico predictions.

4 Drug–Drug Interactions

4.1 Cytochrome p450 Inhibition

One of the key assumptions regarding the patient population for most new therapeutic agents is the great likelihood of polypharmacy [6]. Because concomitant medications may share common distribution, metabolism, or elimination pathways, the possibility exists that changes in pharmacokinetics of a given agent could be induced by co-administration of other molecules, producing a drug–drug interaction that can impact safety or efficacy. Therefore, with few exceptions, the target product profile for a new drug will include a requirement that the agent not produce clinically significant drug–drug interactions. Working from the knowledge that many problematic drug–drug interactions in recent history have been related to interference with oxidative metabolism mediated largely though CYP450 isozymes [7], most modern lead optimization programs operate with a primary goal of selecting a lead molecule without such a liability. To this end, relatively high-throughput in vitro assays have been developed to identify compounds that are potent inhibitors of CYP450 isozymes commonly involved in drug metabolism (typically CYP1A2, CYP2C9, CYP2C19, CYP3A4, and CYP2D6) [8]. For screening purposes, such assays usually rely on interference by the new chemical entity with the CYP450-dependent metabolic conversion of a known pro-fluorophore into its fluorescent state, generally run in at least a 96-well plate format. Because fluorescence detection of a fixed pro-fluorophore is used, these assays can be applied without the need for bespoke bioanalytical assay development for each

new chemical entity of interest [9]. As a result, these assays often are invoked quite early on a team critical path, often just after or even in parallel with in vitro pharmacologic potency. In fact, numerous companies are screening all new compounds synthesized as part of inherent profiling of their chemical libraries.

The increase in throughput afforded by such assays has undoubtedly played a major role in reducing development attrition due to clinically significant drug–drug interactions. However, in a lead optimization setting, such assays cannot be used blindly. For instance, one important consideration is that the assay output is quite sensitive to slight inter-laboratory variations in assay format (enzyme source, amount of protein in the assay, solvent used for the compound of interest, etc.). Therefore, although the generation of IC50 values for many new molecular entities is possible, to provide any meaningful output the assays must be placed into more detailed context. Clinically significant drug–drug interactions will be dependent not only on absolute inhibitory potency of a test agent, but also upon the concentrations of that agent achieved in the clinic. To be more specific, the relevant concentration is that at the active site of the enzyme, however, since these data are not readily available, plasma concentrations or even simply the administered dose are often used as a surrogate. This relationship must be explored for the individual assay being invoked in lead optimization by validating the assay against molecules known to produce clinically significant drug–drug interactions and producing a plot such as the one shown in Fig. 3. For this hypothetical assay, it is clear that regardless of clinical dose, molecules with an IC50 <1 μM against one of the major CYP450 enzymes will have a high probability of exhibiting a clinically significant drug–drug interaction, and those with IC50s above 10 μM have a low probability of such issues. For compounds with IC50s between 1 and 10 μM, compounds with a large clinical dosage are somewhat more likely to cause a drug–drug interaction issue than those with low clinical dosages. For an assay with this response for molecules with known clinical interaction potential, one could easily envision setting meaningful "go/no-go" screening criteria for new chemical entities evaluated in the assay. However, only by constructing such a plot for the specific assay and conditions being employed one can arrive at a meaningful interpretation of the IC50 values obtained, and the medicinal chemist should insist on the development of such a relationship prior to the establishment of pass/fail criteria for a CYP450 inhibition screen.

Obviously, it would be preferable to select a lead compound free from potential drug–drug interaction issues. However, depending on the chemotype being optimized, it may not be possible to separate potency against relevant CYP450 isozymes and activity at the biological target of interest. In these instances, a team may elect to progress a molecule through development in the anticipation that the dosage will be low, or in the hope that it will represent one of the instances where the in vitro IC50 is not representative of in vivo drug–drug interactions and test this hypothesis in the clinic. Short of this, or to help further mitigate or inform the risk of this approach, it may be reasonable to test such a molecule in a preclinical in vivo model of drug–drug interactions, such as nonhuman primates co-dosed with a CYP450 inhibitor [10]. Another potential means to assess the in vivo significance

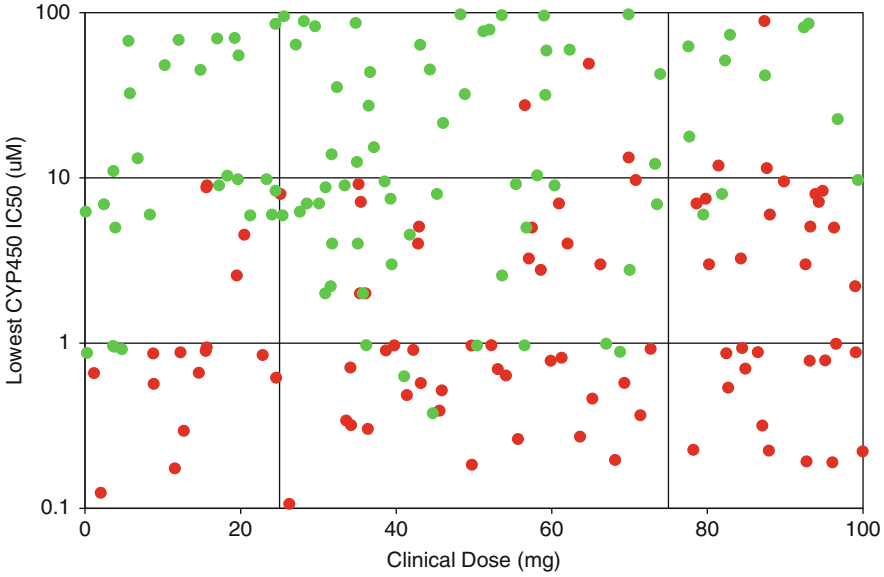

Fig. 3 This plot illustrates an approach to the practical validation of an in vitro CYP450 inhibition assay. *Red symbols*, representing molecules which have demonstrated a clinically significant drug–drug interactions, clearly predominate with higher dose and lower IC50s in the hypothetical assay. Because of interassay variability, such a relationship should be constructed for any CYP450 assay employed on a team's critical path

of a low CYP450 IC50 would be to conduct a preclinical pharmacokinetic linearity experiment [11]. If a molecule exhibits pharmacokinetic nonlinearity at relatively low concentrations in vivo, it may be more likely to be subject to clinically significant drug–drug interactions. Such studies are somewhat labor-intensive and are not generally routine in a lead optimization paradigm; however, they may be useful to help offset the risk of progressing a molecule with significant in vitro CYP450 inhibition liabilities.

4.2 Cytochrome P450 Induction

Although not typically a major area of resource expenditure in drug discovery lead optimization programs, the issue of CYP450 induction should also be addressed. CYP450 induction can occur when a molecule either directly or indirectly activates gene expression of drug metabolizing enzymes, resulting in increased metabolic capacity. Although not a common phenomenon, clinically significant drug–drug interactions can be attributed to CYP450 induction, rather than inhibition [12]. Given the relatively low frequency at which this tends to occur compared to drug interactions mediated by inhibitory processes, many lead optimization programs do

not tend to attempt to screen for this activity. However, in some instances, considering a mechanistic screen for this property may be reasonable. For instance, it has been determined that induction of the nuclear receptor PXR is one of the primary mechanisms for induction of CYP450 activity [13]. PXR interaction assays have been designed and may be deployed in a drug discovery team. Such assays may be particularly important if the molecular target of a discovery program is also a nuclear receptor, as it would then be more likely that PXR interaction could be an important off-target effect of molecules in that program. However, for such an in vitro assay, the same caveats apply as described above for a CYP450 inhibition assay – until a given induction assay is validated using tool molecules with and without clinically significant drug–drug interaction potential, substantial caution must be employed in their use. As another level of interrogation of induction reactions, important data can be derived from the initial nonclinical safety studies with new molecules. Often, increases in liver weight and/or decreases in drug exposure with chronic dosing can be indicators of CYP450 induction. Further, livers from these studies can be harvested and subjected to analysis to determine abundance or activity of key CYP450 isozymes of interest (provided, of course, that the same level of assay validation as described above, with compounds known to produce clinically significant CYP450 induction, has occurred). However, these analyses are less useful in lead optimization since (1) the results from the nonclinical safety species must be extrapolated to humans; (2) the dosages used in the nonclinical safety studies are likely high multiples of those that will be encountered in a clinical setting; and (3) by the time a team progresses to nonclinical safety studies, failure to identify a real CYP450 induction issue until this time would represent a significant issue in terms of time lost in the development process.

4.3 Transporter Substrate/Inhibitor Status

As a final note on drug–drug interactions, significant effort has been expended in roughly the past decade to select molecules without CYP450 interaction liabilities. Medicinal chemists have successfully identified numerous structural motifs that frequently produce CYP450 interactions [14] and often tend to avoid these functional groups when designing new molecules. It can be argued that such selection has exerted an "evolutionary pressure" on chemical libraries and on drug candidates which reduces the risk for drug–drug interactions mediated by oxidative metabolism, but which also selects for molecules that are eliminated by other means, such as by metabolism via conjugation reactions (Phase II metabolism) or by elimination intact via excretion through active transport processes (Phase III reactions). Such disposition mechanisms can also be the source of clinically significant drug–drug interactions, and there is growing attention on this area of research. It is widely recognized that drug transporters are involved in a nearly ubiquitous manner in drug disposition [15], including uptake and efflux in the major absorption and extraction organs of the intestine, liver, and kidney [16–19], as well as distribution into key organs of effect or side effect, such as the brain [20].

Given their importance in drug disposition, it is not surprising that screening of new chemical entities for their interaction with various drug transporters has made its way into drug discovery lead optimization critical path screening. However, such an attempt is often met with several challenges. First, the multiplicity of drug transporters, each of which has varying degrees of substrate specificity, tissue distribution, and reaction kinetics, is frankly overwhelming. To date there is no possible means to screen for interactions with all possible relevant transporters in a drug discovery setting, given the inherent resource and time constraints on the process. Given this problem, there has been a major focus on a few key transporter proteins, particularly P-glycoprotein. However, even screening for interaction with a single protein is not straightforward, different methodologies often give different "answers" to the question of whether a given molecule is a substrate or inhibitor of P-glycoprotein, and a suite of assays may be required to get a thorough understanding [21]. A second major issue with screening for interactions with transporters in a discovery setting is that it is difficult to find animal models to confirm in vivo the relevance of any in vitro findings. Animal models used to study transport processes have been generally limited to rodents, due to their accessibility and genetic manipulability, and the work that has been performed in nonrodents has indicated functional differences in transporter activity [22, 23]. Even the rodent work that has been done has been met with challenges, not the least of which is that functional perturbation of transporter systems via genetic variants or targeted genetic manipulation appears to be often met with the biochemical response of modulation of compensatory transporter and enzyme activities, complicating any data interpretation from such experiments [24–26].

Another issue with incorporating assays for transport interactions in drug discovery is that even using a well-designed assay system that provides a reasonably clear experimental answer, the functional significance of such a finding is not completely understood. For instance, P-glycoprotein is well known to be highly expressed and active in the intestinal epithelium as well as at the hepatic canalicular membrane [27]. This anatomical placement, as well as its broad substrate specificity and early experimental data, has led many investigators to conclude that molecules that are substrates for P-glycoprotein can be expected to be more likely to demonstrate low oral bioavailability, as they may be subjected to intestinal efflux as well as first-pass hepatic biliary excretion. Indeed, numerous examples of molecules that are P-glycoprotein substrates with low oral bioavailability can be cited. However, this relationship does not globally hold true. Table 1 displays a number of drugs that would be presumed to be subject to fairly extensive efflux transport, as evidenced by a permeability value >5-fold higher in the basolateral-to-apical direction (efflux) than in the apical-to-basolateral direction (absorption) [27–29]. However, all of these molecules demonstrate reasonable human oral bioavailability, with many demonstrating >50% oral bioavailability. On the whole, then, given the complexities of the various assays and the difficulties in extrapolating to meaningful clinical outcomes, it would be difficult to justify expending substantial resource screening for transporter interactions in a drug discovery critical path. One exception to this general statement would likely be in the area of drugs intended to target central

Table 1 Comparison of human oral bioavailability values with in vitro bidirectional permeability values, demonstrating that high apparent in vitro efflux does not always correlate with low human oral exposure

Molecule	B:A/A:B ratio	Human %F
Domperidone	31	15
Dipyramidole	23	27
Cyclosporin A	6	28
Tacrolimus	5	30
Verapamil	8	30
Losartan	31	36
Eletriptan	32	50
Etoposide	12	52
Nelfinavir	9	55
Clarithromycin	31	55
Cimetidine	5	60
Digoxin	8	60
Ritonavir	25	60
Amprenavir	28	60
Trimethroprim	50	60
Furosemide	10	61
Indinavir	15	65
Cetirizine	6	70
Dicloxacinnin	32	70
Quinidine	27	75
Fluvastatin	5	79
Dexamethasone	12	80
Prednisolone	10	82
Levofloxacin	5	99

Data derived from several references [27–29]

nervous system diseases. The link between P-glycoprotein substrates and poor brain penetration appears to be stronger than that for oral absorption properties, and screening for transporter status would likely be an important component of lead optimization for such programs [30].

5 Undesirable Pharmacokinetic Properties

The vast majority of new therapeutic agents are developed with the intent of delivery via oral administration. For such molecules, successful delivery to the molecular site of interest requires adequate absorption from the gastrointestinal tract into the bloodstream, sufficiently low clearance and first-pass hepatic extraction to allow entry into the systemic circulation, an appropriate tissue distribution profile that allows penetration into the target tissue, and a half-life long enough to allow infrequent (ideally once-daily) dosing but not so long as to result in prolonged exposure and toxicity. This balance of properties can be as difficult to attain as it may sound on the surface, and lead optimization teams often spend a majority of their time during lead optimization attempting to obtain acceptable pharmacokinetic

properties. The problem is further compounded by the fact that for new chemical entities, data in the model system of interest (humans) cannot be readily obtained, and the chemist and DMPK scientist are left with the use of a variety of imperfect model systems in which to optimize lead compounds in the belief that these efforts will ultimately lead to a molecule with acceptable clinical pharmacokinetic properties.

5.1 In Vivo Versus In Vitro Test Systems

Probably the most effective means by which to test new compounds for their pharmacokinetic properties is by screening in vivo in preclinical species. This is a somewhat controversial statement and will by no means be agreed by all scientists. Indeed, the ethical concerns surrounding in vivo testing are real, and in vivo screening can be a costly endeavor. However, it can be argued that the value of the derived data is unmatched by any in vitro system, which at most can be supportive of an in vivo screening approach. For instance, many companies use an in vitro membrane permeability screen to categorize molecules with respect to their ability to cross biological membranes, as a surrogate for the prediction of in vivo absorption. The predictive reliability of such systems has been reviewed elsewhere [31]; however, even a reliable in vitro membrane permeability system suffers from the disadvantage of questionable predictivity for substrates for active transport processes (either efflux or uptake transporters) and for molecules subject to extensive trans-intestinal metabolism. Further, the use of such assays generally requires the development of analytical methodologies specific to each compound of interest, and for only a small amount of additional resource, in vivo absorption data can be obtained in a preclinical species. Likewise, similar issues are encountered with in vitro screens for rates of metabolic clearance. Although some successes have certainly been noted, at best it can be said that the reliability of the predictions obtained from in vitro clearance screens will vary by structural series. Therefore, to establish whether an in vitro intrinsic clearance screen will be predictive for a given chemical series, one must first assay numerous molecules in a given series with a variety of in vitro and in vivo clearance rates to determine sensitivity and specificity of the in vitro clearance rate prediction in a preclinical species. For instance, if rat in vitro clearance is unable to accurately rank order or quantitatively predict rat in vivo clearance, then the use of a human in vitro clearance screen to select lead compounds with appropriate human clearance properties should be seriously questioned. In the author's experience, it is rare (though not unprecedented) to encounter a chemical series with a useful and simple correlation between predicted clearance from an in vitro screen and actual in vivo clearance. Often, when such correlations are found, the predictive ability of the in vitro screen is only attained after correction for such parameters as binding to the subcellular matrix and to plasma proteins [32]. Furthermore, the bioanalytical challenges of supporting in vitro clearance screens should not be underestimated. Overall, one could postulate that given the validation that should be performed on such screens, the multiple pieces of data often required to achieve predictivity, and the low rate of success with such

systems, it would be simpler to exclude such assays altogether. Regardless, the use of such data must be viewed with caution and supported by sufficient validation to ensure that misleading results are not being obtained.

5.2 Applicability of In Vivo Preclinical Approaches

The above discussion should not be interpreted to imply that in vivo preclinical pharmacokinetic assays are trouble-free and perfectly predictive. Indeed, animal data are obviously not completely equivalent to results in humans, and some consideration should be given to questions related to cross-species correlations. Very broadly speaking, there are three key parameters to be considered when extrapolating pharmacokinetic parameters from preclinical species to humans: volume of distribution and clearance, which drive half-life and therefore duration of exposure, and oral bioavailability, which determines the extent of exposure [33]. In general, human volume of distribution is reasonably well predicted by direct extrapolation from in vivo preclinical studies, regardless of species used [34]. From a mechanistic perspective, this predictivity is somewhat intuitive, since volume is driven primarily by passive processes that are inherent to a given molecule, rather than active processes that are likely to vary across species. Some exceptions certainly exist, such as molecules for which distribution is driven by active transport processes, protein binding, or receptor binding. However, in general volume can be usually taken as a reasonable constant across species for a given molecule.

The prediction of human clearance is more problematic. As noted above, in vitro models for predicting clearance are widely used, but their predictivity is questionable. In a typical lead optimization cascade, as illustrated in Fig. 2, rodent pharmacokinetics (usually in the rat) are used as a first-pass approximation of whether a molecule will demonstrate generally acceptable pharmacokinetics. However, rats are usually efficient eliminators of xenobiotics, and predicting human clearance from rat data is not particularly successful [35]. For instance, it has been demonstrated using a structurally diverse dataset that when a high clearance value is obtained in the rat, particularly with molecules with cLogP values >3.0, human clearance is more likely to be low or moderate, rather than high [36]. This raises an interesting dilemma – since the rat is a common species of first choice in pharmacokinetic screening, and since modern chemical libraries are well stocked with lipophilic molecules, it is possible that many compounds are discarded because of high rat clearance, when in fact they may demonstrate perfectly acceptable human clearance values. In contrast, it seems clear that prediction of human clearance based on in vivo clearance in the nonhuman primate is a reasonably predictive approach in most instances [35]. Of course, monkey screening studies carry their own challenges and may be beyond the resource reach of many organizations. However, it seems likely that by judicious use of nonhuman primates, coupled with more knowledgeable interpretation of rodent data [36], in vivo screening

approaches can provide a reasonable qualitative, if not quantitative estimate of human clearance.

With respect to oral exposure, similar challenges exist. Since clearance (particularly first-pass clearance) is a major determinant of overall exposure, all of the issues with predicting human clearance are stacked upon separate issues with predicting human absorption properties when evaluating the likelihood of a new molecule to demonstrate acceptable human pharmacokinetics. As is the case with clearance prediction, prediction of human oral exposure based on monkey oral exposure data tends to be the best predictive model [37]. However, when human exposure is predicted incorrectly from monkey data, it tends to be under-predicted. Again, this raises the possibility that molecules which could demonstrate acceptable human pharmacokinetics could be discarded when using this kind of assay in a critical path paradigm. Certainly such assays are useful to reduce attrition, however, along the way there is the possibility that potentially successful molecules could be eliminated from consideration. However, with that said, it should be noted that regardless of predictivity, it is generally useful to work with molecules with acceptable preclinical pharmacokinetic properties. For instance, the rat is a key species for use in nonclinical safety assessment, and even if one mechanistically could justify developing a molecule with poor rodent pharmacokinetics based on likely human performance, having a molecule for which the generation of reasonable exposure profiles in the rat is difficult will result in problematic preclinical development, adding time and logistical difficulty to the development process.

With respect to the issue of throughput when using an in vivo assay, it is relatively straightforward to screen dozens of compounds on a weekly basis for in vivo pharmacokinetic properties, as long as the supporting in vivo scientific group is adequately staffed. While not the same throughput as could be achieved in an in vitro 96-well-format assay, this magnitude of screening is usually sufficient to meet the needs of even a well-resourced lead optimization effort. Numerous approaches are available to increase in vivo throughput, including classical cassette or N-in-1 dosing [38], the so-called rapid-rat approach [39], and sample pooling, in which the pharmacokinetic experiments are performed on individual molecules but the incurred samples are pooled for subsequent bioanalysis [40]. Each option still results in a substantial bioanalytical workload, and having well-equipped bioanalytical chemists with automated sample preparation tools and adequate tandem mass spectrometers is key to these approaches.

5.3 Approaches to Explore Absorption and Clearance Issues

In addition to the whole-body pharmacokinetic approaches outlined above, at times it may be useful to perform more detailed mechanistic studies to resolve pharmacokinetic issues. For instance, during the course of a lead optimization program a team may find that it has an apparent absorption issue – low oral bioavailability in an animal species coupled with low to moderate clearance. In addition to the

somewhat random "brute force" approach of iterative structure–property relationship generation to work through the problem, one possible line of investigation to consider would be to study a series of exemplar compounds to attempt to understand what chemical features contribute to the poor absorption properties. Numerous in vitro models are available to study basic absorption properties – artificial membrane permeability assays, cultured epithelial monolayers, Ussing chamber experiments using segments of isolated intestine, and others [41]. However, often the best approach in this situation is once again to use the intact animal model in which the pharmacokinetic issue arises. Models for isolating and collecting samples from the hepatic portal circulation and the systemic circulation have been described in all of the major preclinical species, and these models are no less onerous to establish than properly validated forms of the in vitro studies described above.

Likewise, in instances where a lead optimization program encounters issues with high clearance, it is usually tempting to attempt to invoke an in vitro clearance screen to resolve the issue. Such assays are relatively easy to perform and require relatively little supply of compound. However, as mentioned above, the results from such screens can be problematic, as there are numerous experimental and data interpretation complexities that can influence the results. For instance, there is large inherent variability (up to twofold) in human microsomal protein content, up to nearly threefold interindividual variability in hepatocellular content, interindividual variability in cytochrome P450 activity, and significant batch-to-batch variability in microsomal activity from different vendors [42–44]. In addition to these experimental preparation issues, the experimental conditions themselves have a large bearing on the data generated, including incubation time and protein concentration in the incubation, nonspecific matrix binding, and solvent composition [45–49]. As would be expected for an assay with so many parameters driving the outcome, a survey of the literature revealed profound (10s to 100s-fold) intra-laboratory variability for in vitro intrinsic clearance observations even for well-characterized test compounds [50]. In these instances, retrospective analysis usually provides some mechanistic rationale for such experimental variability and can often even suggest some combination of mechanistic scaling factor that results in a successful prediction. However, in a drug discovery setting, one does not have the luxury of knowing the "answer" (human clearance) for comparison with the predicted outcome from an in vitro assay. Accordingly, the use of in vitro data, even to solve limited problem sets around clearance issues, must be adopted with caution.

Although the above discussion highlights the risks and limitations involved with in vitro metabolism screening, and these issues must be kept foremost in mind, there are instances where in vitro screening can be a useful directional tool in a lead optimization program. For example, there may be instances where a given chemical series has been shown through in vitro–in vivo validation studies to lend itself to reasonable clearance predictions via screening in vitro for metabolic turnover rate. In these cases the in vitro screen can afford a useful and rapid means for screening new compounds for evidence of improvement in metabolic clearance. Also, in vitro metabolism systems can be useful in determining the major metabolic pathways for a set of exemplar compounds in a chemical series, since incubation in vitro and

isolation of metabolites is more straightforward than similar in vivo studies. Once a route of metabolism is postulated, it may be possible to better design chemical modifications intended to exploit metabolic "hot spots." Of course, numerous caveats to this approach apply, not the least of which is that quite often the blocking of a putative site of metabolism on a molecule simply shifts the site of metabolism to a different moiety. However, in instances where straightforward in vivo pharmacokinetic lead optimization is not yielding progress, and where resource allows, in vitro studies may provide an adjunct to the in vivo approach.

5.4 Lead Optimization in Humans: The Microdosing Approach

No discussion of pharmacokinetic optimization would be complete without some consideration of testing in the ultimate species of interest, humans. Historically the use of humans to perform "lead optimization" has been far too costly and time consuming to even consider using in an iterative manner. However, in recent years there has been growing interest in the use of the so-called microdosing approaches, in which single subpharmacological doses of new chemical entities may be administered to humans with a less extensive toxicology data package than ordinarily required for a standard IND application [51, 52]. Coupled with the use of ultrasensitive bioanalytical techniques such as accelerator mass spectrometry, the concept is that human pharmacokinetic studies can be conducted to help select the most optimal amongst several lead candidates, or to rapidly identify molecules with poor pharmacokinetic properties and preclude further study. The concept is intriguing, and there are likely many examples of development programs that will benefit from such an approach. However, several caveats must be borne in mind when contemplating such an approach. First, although the use of human microdosing may allow a team to perform less resource-intensive "no-go" decision making, if the result of the study suggests a "go" decision, then the team must return to the preclinical development stage and prepare the full CMC and toxicology study package required for full clinical development (or work on these attributes in parallel). As a result, for programs that ultimately move forward, the use of the microdosing approach can actually cost, rather than save, time and money. Therefore, the approach is best invoked with programs with a low probability of clinical pharmacokinetic success. Second, the validity of the pharmacokinetic data obtained with microdosing can be questioned. One can readily envision scenarios in which microdosing would yield a "false positive" signal, such as if the absorption of a molecule is solubility limited, and the micro-dose is absorbed in a quantity that will not scale linearly with dose. Alternatively, "false negative" signals can also be obtained, as might be the case with a substrate for an intestinal efflux transporter which would be readily saturated with a clinically relevant dose, but which efficiently effluxes the micro-dose back into the intestinal lumen. Therefore, in these instances, there may be scenarios in which the human microdose data are not as predictive of the human response as a preclinical species at a higher dose. One way to possibly guard against these issues could be to

perform a sort of validation study using a microdose in a preclinical species for the molecule of interest compared to a clinically relevant dose. Depending on whether dose-proportionality is observed in the preclinical species, more or less confidence may be gained in the clinical microdose approach [53].

6 Additional Pharmacokinetic Properties Driving Efficacy and Safety

6.1 Plasma Protein Binding

As noted above, it is clear that pharmacokinetics influences safety and efficacy via concentration-versus time profiles and the potential for drug–drug interactions. However, there are additional parameters traditionally measured in a pharmacokinetic lead optimization paradigm that may also address efficacy or safety risks. One such parameter of interest is plasma protein binding. Although most standard bioanalytical methodologies in pharmacokinetic lead optimization programs are designed to measure total drug content in a given sample, usually after protein denaturation and precipitation, the total drug amount is only one component of the overall picture. Drugs in plasma associate in both a specific and nonspecific manner with various proteins in plasma, most notably albumin, α1-acid-glycoprotein, and various lipoproteins [54]. Although these interactions are usually reversible, the kinetics of dissociation may be slow compared to rate of metabolism or elimination, and therefore the free fraction of drug can be an important determinant of efficacy. The relationship between free fraction and efficacy has been clearly elucidated in certain therapeutic areas, such as anti-infective therapy [55] or CNS-active drugs [56]. However, protein binding can play a role in efficacy in any therapeutic area and should be considered during lead optimization in drug discovery [57].

There are several major methods available for determining protein binding, including ultrafiltration, equilibrium dialysis, and ultracentrifugation [58]. Although a detailed review of each of these methodologies is beyond the scope of this chapter, it is critical that the discovery scientist recognize that none of these methods is ideal, and that each suffers from serious potential drawbacks. For instance, most available ultrafiltration devices are subject to very high nonspecific binding to the device for many molecules, rendering them inappropriate for protein binding determination. Anecdotally, in the author's experience, ultrafiltration has proved suitable for $<10\%$ of the drugs attempted to be measured by this technique. Consequently, many scientists now consider equilibrium dialysis to be the method of choice for protein binding determinations. However, to obtain an accurate measure of free fraction, there are a number of experimental considerations to determine, including time to equilibrium and stability and recovery from the equilibrium dialysis device. Many molecules will also demonstrate poor recovery from the device, or do not attain equilibrium within a reasonable timeframe to avoid plasma protein degradation.

Furthermore, the bioanalytical matrix in an equilibrium dialysis experiment varies with time; the "receiver" chamber starts as simple buffer, but over the course of dialysis the movement of ions and other small molecules in the plasma on the "donor" side of the device contaminates the receiver chamber and can often significantly alter the matrix so as to produce a differential ion suppression effect in mass spectrometric detection of the analyte of interest [59]. For ultracentrifugation, the lack of a filter in the system can often improve overall recovery; however, the formation of a lipid layer at the top of the ultracentrifugate and partitioning of lipophilic compounds into this layer is problematic.

Given these difficulties with determining a numerical value for protein binding, and the fact that none of these methods includes a means to assess actual binding affinity rather than capacity [60], it would be desirable to identify alternative means of assessing the impact of protein binding. The first place where problematically high plasma protein binding often presents itself is during the initial pharmacokinetic characterization of a new compound. If protein binding is sufficiently high, the molecule may not be available for distribution outside the vascular space and may also not be available to be cleared by the liver or kidney. Therefore, in instances where volume of distribution is at or below that of the vascular space ($<$0.1 L/kg), and clearance is very low ($<$1% of liver blood flow), high plasma protein binding should be suspected as a contributing factor. Another practical approach to evaluating the relevance of tight plasma protein binding is functional efficacy assays. It can often be quite beneficial to perform in vitro efficacy studies in the presence and absence of exogenous protein whenever possible; profound shifts in activity under the different conditions suggest problematic protein binding. Not all in vitro assays will be amenable to the addition of exogenous protein, and in these instances the best indication of whether protein binding will be an issue may be in an in vivo model of efficacy. Fairly sophisticated approaches can also be adopted to correlate protein binding with tissue distribution and efficacy [61]; although these are not usually readily applicable to screening methodologies, such approaches can be very useful to probe the role of protein binding for an exemplar compound in a given line of chemistry.

6.2 *Volume of Distribution*

Another pharmacokinetic parameter often measured but sometimes not acted upon is volume of distribution. Volume of distribution is a composite parameter that can be mechanistically driven by a number of different factors, including protein binding, rate of distribution out of the vascular space into tissues, and binding to specific components of tissue. As noted above, very low volumes of distribution (at or below vascular volume) can be suggestive of high plasma protein binding that may restrict efficacy. In addition to these very low volumes of distribution, even "reasonable" volumes may reflect a tissue distribution that will restrict efficacy. Total body water is generally assumed to be equivalent to 0.6–0.7 L/kg; therefore, volumes less than this value suggest that the drug may not be leaving the

extracellular space. Further, for many disease targets, larger volumes of distribution may be desirable, as this would suggest more extensive distribution into one or more tissues that could be pharmacological targets. The converse of this is also true; as volume of distribution increases, and tissue distribution likely increases, it becomes increasingly likely that concentrations in tissues of toxicological concern may reach values that can be associated with unwanted side effects. There are no generally accepted guidelines for striking this balance between tissue distribution for efficacy and safety, and although there is no commonly agreed "bad" value for a volume of distribution, it can be assumed that when volume approaches or exceeds a value of 5–10 times total body water (>3.5–7 L/kg), substantial tissue concentrations are being achieved. Depending on the particular mechanism of action and therapeutic window of a given test agent, a balanced approach must be employed to optimize volume of distribution.

6.3 Metabolism to Reactive Species

Finally, the role of reactive metabolites as a pharmacokinetic event that can be a key determinant of drug safety should be mentioned. A full treatment of this subject will be given in a separate chapter in this volume. However, it is important to note here that in the course of lead optimization, one of the more effective "pharmacokinetic" activities that can be performed to reduce toxicology-related drug attrition is to perform a screen for reactive drug metabolites. The biotransformation of a parent molecule to a metabolite with reactive properties is a major risk for downstream safety issues, whether in the form of antigenicity or genotoxicity [62]. Certainly more definitive metabolic characterization of the selected candidate molecule(s) will be conducted later in development, along with more detailed ability to conduct risk assessments around any reactive metabolites generated. However, during lead optimization, one actually has the ability to "fix" the problem altogether, by designing out the molecular feature(s) in the molecule associated with reactive metabolite formation. There are a number of pharmacophores that have a known propensity for reactive metabolite formation [63], and chemists should avoid these moieties if possible. However, in the event that balancing the other required developability properties within a given series requires using one of these potentially problematic pharmacophores, as well as to flag issues with novel chemotypes, it is generally prudent to incorporate an assay for reactive metabolites during lead optimization. There are a number of different potential ways to detect formation of such metabolites, including the covalent binding of radioactivity in an in vitro metabolic incubation or in an in vivo disposition study, or the use of a so-called glutathione trapping screen which relies on the binding of reactive moieties to excess glutathione in an in vitro incubation [64]. Another fairly straightforward way to detect reactive metabolites is to perform an in vitro metabolic incubation in the presence of hepatocytes, and subject the resultant incubation mixture to mass spectrometric analysis. Detection of mass fragments such as parent

mass + 34 AMU or parent mass + 323 AMU can suggest arene oxide formation, or arene oxide formation and subsequent glutathione conjugation; use of exact mass data on a high resolution mass spectrometer can provide additional certainty around the identify of such mass shifts [65, 66]. Although such findings in a discovery setting are far from definitive and would not represent an absolute no-go finding, any work that could be done to select an alternate candidate molecule in these situations would aid in reducing downstream attrition.

7 The End Game: Modification of the Critical Path and Candidate Declaration

As depicted in Fig. 2, the contents of this chapter have addressed many of the components of a "standard" lead optimization screening cascade leading to candidate selection. However, perhaps as important as understanding how to create and follow such a screening tier is recognizing that there are exceptions to every rule, and that during the life of a lead optimization program there will be instances where the established critical path should be violated or altered. Some examples of this described above include instances where acceptable CYP450 inhibition profiles cannot be attained after extensive iterative chemistry, demonstration of chemical structure–activity relationships which suggest that the preclinical species may not be predictive of human pharmacokinetics, or availability of straightforward in vivo pharmacology models that may replace other developability criteria such as protein binding or pharmacokinetic evaluation. Another instance where excursions from the critical path may be valuable would be "reality checks" to confirm that sometimes extensive effort being expended to improve in vitro developability parameters or rodent pharmacokinetics is not directing the chemistry towards failure in a later assay, such as nonrodent pharmacokinetics, reactive metabolite formation, or toxicology.

Additionally, while the generic critical path is representative for straightforward oral drug programs for systemic disease, other treatment modalities will require different critical paths. For instance, in an oncology program designed to administer a likely cytotoxic agent via intravenous infusion, considerations of drug–drug interactions often become less important, and it becomes critical to identify molecules with high clearance and relatively short half-lives, so that controlled drug exposure can be more readily achieved. Likewise, the development of topical therapeutics usually includes in vitro and in vivo assays designed to determine dermal penetration and residence time, while aiming for high systemic clearance for any drug that "leaks" into the systemic circulation. Ophthalmic drug development programs are another example requiring atypical critical paths; depending on the route of administration (topical, subconjunctival, intravitreal) particular assays will be required to optimize new chemical entities for these target product profiles. Obviously it is critical for the discovery scientist to be fully aware of the eventual commercial requirements of a given product to allow appropriate critical path design.

With respect to the ultimate declaration of a candidate molecule, or "how good is good enough," the commercial target product profile is a useful and important start. At the inception of a lead optimization program, the team should certainly have a solid understanding of the product requirements, and the window of acceptability around those criteria. For instance, if achieving once-daily dosing is an absolute commercial go/no-go feature, potential candidates may be viewed differently than if once-daily dosing is a "nice-to-have" attribute. Certainly the team should prepare and agree on specific assay criteria that will be met for a given candidate, and in many cases these may be pre-defined by general corporate guidelines (i.e., minimum bioavailability in preclinical species; maximum percent inhibition of CYP450 enzymes; etc.). However, it is rare that a candidate achieves every aspect of the desired profile. Given the process of balancing multiple desired properties (some of which often require chemical changes that act in opposition to one another), the lack of complete certainty that a given preclinical assay will be absolutely predictive of the human response, and the desire to move programs forward as quickly as possible, in the end the candidate selection decision must be a risk-reward balance decision. As a result, the decision point often becomes as much about organizational resource and risk tolerance as about the pure science behind the potential candidate. Discovery scientists should also endeavor to educate themselves about these nonscientific criteria within their organizations and understand their role in the decision-making process.

The successful execution of a lead optimization program in drug discovery requires a carefully balanced perspective. There can be a huge number and variety of different types of assays from which to select for a critical path, and following them all would doubtless lead to near-paralysis in decision making and a huge investment in resource. It falls therefore to the drug discovery professional to survey the available landscape of assays in comparison with the team's target product profile and molecular mechanism of action to design an effective and efficient critical path. It has been the aim of this chapter to highlight some of the areas of concern in this selection process and to enable team leaders to thoughtfully analyze the possible options for their programs and select accordingly.

8 Conclusions

The successful execution of a lead optimization program in drug discovery requires a carefully balanced perspective. There can be a huge number and variety of different types of assays from which to select for a critical path, and following them all would doubtless lead to near-paralysis in decision making and a huge investment in resource. It falls therefore to the drug discovery professional to survey the available landscape of assays in comparison with the team's target product profile and molecular mechanism of action to design an effective and efficient critical path. It has been the aim of this chapter to highlight some of the areas of concern in this selection process and to enable team leaders to thoughtfully analyze the possible options for their programs and select accordingly.

References

1. Frank R, Hargreaves R (2003) Nat Rev Drug Discov 2:566
2. Ekins S, Waller CL, Swaan PW, Cruciani G, Wrighton SA, Wikel JH (2000) J Pharmacol Toxicol Methods 44:251
3. Lipinski CA, Lombardo F, Dominy BW, Feeny PJ (1997) Adv Drug Deliv Rev 23:3
4. Veber DF, Johnson SR, Cheng H-Y, Smith BR, Ward KW, Kopple KD (2002) J Med Chem 45:2615
5. Stouch TR, Kenyon JR, Johnson SR, Chen X-Q, Doweyko A, Li Y (2003) J Comput Aided Mol Des 17:83
6. Steward RB, Cooper JW (1994) Drugs Aging 4:449
7. Huang S-M (2004) Drug-drug interactions. In: Krishna R (ed) Applications of pharmacokinetic principles in drug development. Kluwer Academic/Plenum Publishers, New York, p 307
8. Cupp MJ, Tracy TS (1998) Am Fam Physician 57:107
9. Miller VP, Stresser DM, Blanchard AP, Turner S, Crespi CL (2000) Ann NY Acad Sci 919:26
10. Ward KW, Stelman GJ, Morgan JA, Zeigler KS, Azzarano LM, Kehler JR, McSurdy-Freed JE, Proksch JW, Smith BR (2004) Drug Metab Dispos 32:172
11. Ward KW, Griffiths R, Levy MA, Smith BR (2000) J Pharmacol Exp Ther 293:468
12. Lin JH (2006) Pharm Res 23:1089
13. Dickens M (2004) Curr Top Med Chem 4:1745
14. Woolf TF (ed) (1999) Handbook of drug metabolism. Marcel Dekker, New York
15. Mizuno N, Niwa T, Yotsumoto Y, Sugiyama Y (2003) Pharm Rev 55:425
16. Chandra P, Brouwer KLR (2004) Pharm Res 21:719
17. Dietrich CG, Geier A, Oude Elferink RPJ (2003) Gut 52:1788
18. Chan LMS, Lowes S, Hirst BH (2004) Eur J Pharm Sci 21:25
19. Dresser ML, Leabman MK, Giacomini KM (2001) J Pharm Sci 90:397
20. Kusuhara H, Sugiyama Y (2004) Adv Drug Deliv Rev 56:1741
21. Polli JW, Wring SA, Humphreys JE, Huang L, Morgan JB, Webster LO, Serabjit-Singh CS (2001) J Pharmacol Exp Ther 299:620
22. Ninomiya M, Ito K, Horie T (2005) Drug Metab Dispos 33:225
23. Godinot N, Iversen PW, Tabas L, Xia X, Williams DC, Dantzig AH, Perry WL 3rd (2003) Mol Cancer Ther 2:307
24. Akita H, Suzuki H, Sugiyama Y (2001) Pharm Res 18:1119
25. Oguro T, Kaneko E, Kaneko Y, Numazawa S, Imaoka S, Funae Y, Mikami T, Yoshia T (1996) J Pharmacol Exp Ther 277:1676
26. Schuetz EG, Umbenhauer DR, Yasuda K, Brimer C, Nguyen L, Relling MV, Schuetz JD, Schinkel AH (2000) Mol Pharmacol 57:188
27. Chiou WL, Chung SM, Wu TC, Ma C (2001) Int J Clin Pharmacol Ther 39:93
28. Lin JH, Yamazaki M (2003) Drug Metab Rev 35:417
29. Lin JH (2004) Drugs Today 40:5
30. Summerfield SG, Stevens AJ, Cutler L, del Carmen Osuna M, Hammond B, Tang SP, Hersey A, Spalding DJ, Jeffrey P (2006) J Pharmacol Exp Ther 316:1282
31. van Breeman RB, Li Y (2005) Expert Opin Drug Metab Toxicol 1:175
32. Zuegge J, Schneider G, Coassolo P, Lave T (2001) Clin Pharmacokinet 40:553
33. Gabrielsson J, Weiner D (1997) Pharmacokinetic and pharmacodynamic data analysis: concepts and applications. Apotekarsocieteten, Stockholm
34. Ward KW, Smith BR (2004) Drug Metab Dispos 32:612
35. Ward KW, Smith BR (2004) Drug Metab Dispos 32:603
36. Jolivette LJ, Ward KW (2005) J Pharm Sci 94:1467
37. Ward KW, Nagilla R, Jolivette LJ (2005) Xenobiotica 35:191
38. Frick LW, Adkison KK, Wells-Knecht KJ, Woollard P, Higton DM (1998) Pharm Sci Technol Today 1:12
39. Mei H, Korfmacher W, Morrison R (2006) AAPS J 8:E493

40. Hop CE, Wang Z, Chen Q, Kei G (1998) J Pharm Sci 87:901
41. Borchardt RT, Smith PL, Wilson G (eds) (1996) Models for assessing drug absorption and metabolism. Plenum Press, New York
42. Di L, Kerns EH, Hong Y, Kleintop TA, McConnell OJ, Huryn DM (2003) J Biomol Screen 8:453
43. Wilson ZE, Rostami-Hodjegan A, Burn JL, Tooley A, Boyle J, Ellis SW, Tucker GT (2003) Br J Clin Pharm 56:433
44. Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP (1994) J Pharmacol Exp Ther 270:414
45. Jones HM, Houston JB (2004) Drug Metab Dispos 32:973
46. Kalvass JC, Tess DA, Giragossian C, Linhares MC, Maurer TS (2001) Drug Metab Dispos 29:1332
47. Obach RS (1999) Drug Metab Dispos 27:1350
48. Yin H, Tran P, Greenberg GE, Fischer V (2001) Drug Metab Dispos 29:185
49. Uchaipichat V, MacKenzie PI, Guo X-H, Gardner-Stephen D, Galetin A, Houston JB, Miners JO (2004) Drug Metab Dispos 32:413
50. Nagilla R, Frank KA, Jolivette LJ, Ward KW (2006) J Pharmacol Toxicol Methods 53:106
51. Lappin G, Garner RC (2003) Nat Rev Drug Discov 2:233
52. Wilding I, Garner RC (2004) Good Clin Pract J 11:2
53. Sandhu P, Vogel JS, Rose MJ, Ubick EA, Brunner JE, Wallace MA, Adelsberger JK, Baker MP, Henderson PT, Pearson PG, Baillie TA (2004) Drug Metab Dispos 32:1254
54. Rowland M, Tozer TN (eds) (1995) Clinical pharmacokinetics. Lippincott Williams & Wilkins, Philadelphia
55. Craig WA, Ebert SC (1989) Infect Dis Clin North Am 3:407
56. Pajouhesh H, Lenz GR (2005) NeuroRx 2:541
57. Trainor GL (2007) Exp Opin Drug Discovery 2:51
58. Tillement J-P, Landenlaub E (eds) (1986) Protein binding and drug transport. FK Schattauer Verlag, Stuttgart
59. Annesley TM (2003) Clin Chem 49:1041
60. Wu S, Liu B (2005) BioDrugs 19:383
61. Li Y-W, Hill G, Wong H, Kelly N, Ward K, Pierdomenico M, Ren S, Gilligan P, Grossman S, Trainor G, Taub R, McElroy J, Zazcek R (2003) J Pharmacol Exp Ther 305:86
62. Uetrecht J (2003) Drug Discov Today 8:832
63. Dalvie DK, Kalgutkar AS, Khojasteh-Bakht SD, Obach RS, O'Donnell JP (2002) Chem Res Toxicol 15:269
64. Evans DC, Watt AP, Nicoll-Griffith DA, Baillie TA (2004) Chem Res Toxicol 17:3
65. Samuel K, Yin W, Stearns RA, Tang YS, Chaudhary AG, Jewell JP, Lanza T Jr, Lin SA, Hagmann WK, Evans DC, Kumar S (2003) J Mass Spectrom 38:211
66. Evans CA, Fries HE, Ward KW (2005) Chem Biol Interact 152:25

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The Role of Biotransformation Studies in Reducing Drug Attrition

Douglas K. Spracklin, Amit S. Kalgutkar, and Angus N.R. Nedderman

Abstract Biotransformation has evolved beyond simple structural elucidation of metabolites to provide data that is highly impactful and influential to various aspects of drug discovery. Based on our experience, we have grouped areas where biotransformation-related understanding can make an impact on drug design as follows: (1) defining clearance mechanisms, particularly for drug metabolizing enzymes other than P450s, (2) identifying metabolic hot spots, (3) identifying reactive metabolites, (4) characterizing active metabolites, and (5) assessing metabolite safety. This review will describe how these studies may be used to guide the development of structure–activity relationships to identify and mitigate potential safety liabilities and to interpret pharmacokinetic/pharmacodynamic (PKPD) relationships. Ultimately, a better understanding in all these aspects of drug disposition will aid in reducing candidate attrition.

Keywords Attrition, Biotransformation, Discovery, Metabolism, Safety

Contents

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1 Introduction

Biotransformation is broadly defined as the transformation of chemical compounds within living systems. Understanding the biotransformation of drugs is a key element of the modern drug discovery continuum, both in later stage development and early in the discovery phase. In the pharmaceutical industry, biotransformation as a function is typically practiced by experts and organized as a separate group within the drug metabolism department. Biotransformation is often referred to as a "mature science" because it has its roots in structural elucidation (of metabolites). Because this is a concept familiar to, and of interest to chemists, there is a good understanding and rapport between these two scientific disciplines within the pharmaceutical industry. However, while structural elucidation is a foundational element of biotransformation, scientific advances in related and dependent fields such as enzymology, pharmacology, and analytical methodology have allowed biotransformation groups to provide insights into many different aspects of drug development. As a result, the contributions of biotransformation can extend beyond simple structural elucidation of metabolites, and accordingly, there is a growing desire to include biotransformation scientists as members of drug discovery project teams. Coincident with this, the concern in the pharmaceutical industry with the unsustainable rate of attrition is well documented. Strategies to minimize and identify causes of attrition as early as possible are the norm in pharma today. With biotransformation concepts/understanding related to many aspects of drug development, it follows that biotransformation can play a critical role in understanding and reducing attrition. Based on our experience, we have grouped areas where biotransformation-related understanding can make an impact on attrition as follows:

1. Defining clearance mechanisms
2. Reducing metabolic liability
3. Reactive metabolites
4. Active metabolites
5. Metabolite safety

While these aspects can be applied at any stage of the drug development continuum, this review will emphasize their application in the discovery realm, consistent with the industry desire to identify attrition factors as early as possible.

2 Utility of Biotransformation Studies in Drug Discovery/Development

2.1 Defining Clearance Mechanisms

For the prediction of human pharmacokinetics (PK), much attention has been focused on cytochrome (CYP) P450-mediated metabolism in the discovery of pharmaceuticals. It has been estimated that up to 80% of marketed agents are metabolized by this particular enzyme system [1]. Thus, extensive efforts have been undertaken to set up high throughput screens to enable structure–activity relationship (SAR) development around these drug metabolizing enzymes. However, it is not uncommon to find that as metabolic liability due to P450s is decreased in the course of SAR development, those changes can render the molecules substrates for other enzymes that may not be represented by in vitro systems such as microsomes. Under such circumstances, when examined in vivo, an apparent in vitro–in vivo disconnect is revealed (i.e., greater in vivo metabolism is observed than would be predicted from the in vitro studies). Biotransformation studies can be helpful in resolving these types of discrepancies.

The role of non-P450 enzymes in oxidative metabolism was recently reviewed by Strolin-Benedetti et al. [2]. While older, an equally comprehensive summary has been described by Beedham [3]. Though not an exhaustive list, the non-P450 drug metabolizing enzymes that consistently reveal themselves to us as possible contributors to metabolism of pharmaceutical agents are: (1) monoamine oxidase (MAO), (2) aldehyde oxidase, (3) flavin mono-oxygenase (FMO), and (4) UDP-glucuronosyltransferase (UGT). Unfortunately, in vitro tools for these enzymes that allow for high throughput screening and prediction of human PK are typically not as readily available nor do they possess the fidelity for predicting human PK in a similar way that microsomes do for P450s.

Note: P450-mediated metabolism is clearly an important consideration in synthetic design. For more information, we direct the reader to the literature which is replete with reviews of the basic enzymology, availability of in vitro tools, and general SAR known for the various major P450 isoforms [4–6]. *Because of its comprehensive coverage in the prior literature, and to bring attention to what we view as an underappreciation for other important metabolic enzymes, we have focused this review entirely on non-P450 metabolism.*

2.1.1 Monoamine Oxidase

A search, even limited to reviews, on the topic of "MAO" reveals a plethora of literature; however, the majority of that literature focuses on clinical interest in the use of MAOs used in the treatment of depression and Parkinson's disease. However, the occurrence, multiplicity, distribution, biochemical mechanism, and selectivity of MAO were comprehensively reviewed by Trager [7] and a summary of xenobiotics known, or likely to be metabolized by MAO isoforms (MAO-A

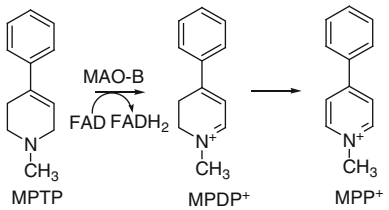


Fig. 1 MAO-mediated aspect of MPTP metabolism

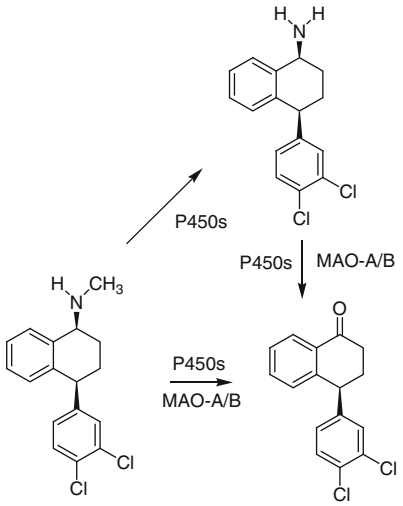


Fig. 2 MAO-mediated aspect of sertraline metabolism

and/or MAO-B) has been compiled by Strolin-Benedetti, Whomsley, and Baltes [2]. Physiologically, MAO is responsible for the oxidative deamination of monoamines such as adrenaline, butylamine, and tyramine [8]. Overall, MAO oxidizes an amine substrate with concomitant reduction of the cofactor flavin adenine dinucleotide (FAD). This is illustrated for the well-documented MAO-B-mediated conversion of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to *N*-methyl-4-phenyl-2,3-dihydropyridinium ion ($MPDP^+$) [9], which is a step in the formation of *N*-methyl-4-phenylpyridine, a well-known neurotoxin (Fig. 1).

With regard to SAR, physiological substrates for MAOs are generally primary amines such as 5-hydroxytryptamine while xenobiotic substrates are also "amine-containing." Considerable effort has been expended in understanding the interactions of the MAO enzymes with their preferred substrates and inhibitors. These efforts and the availability of crystal structures for the two human MAO forms [10, 11] have enabled investigators to delineate in some detail the SAR for both MAO substrates and inhibitors [12]. To illustrate the structural diversity of potential MAO substrates, the MAO-mediated aspects in the metabolism of sertraline, sumatriptan, and propranolol are illustrated below.

Sertraline is an anti-depressant and a member of a class of drugs known as selective serotonin reuptake inhibitors (SSRIs). An in vitro investigation delineating the identities of the various enzymes involved in the metabolism of sertraline has been reported by Obach et al. [13]. Sertraline undergoes deamination to yield sertraline ketone as shown in Fig. 2. These investigators demonstrated that the ketone could be

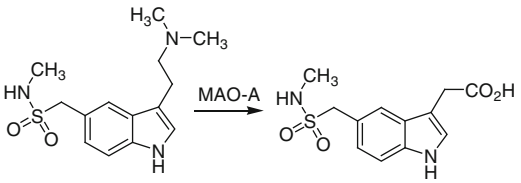


Fig. 3 MAO-mediated aspect of sumatriptan metabolism

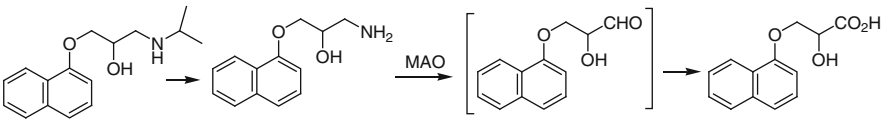


Fig. 4 MAO-mediated aspect of propranolol metabolism

arrived at directly from sertraline, or via the *N*-dealkylated metabolite. While P450s are also involved in these processes, a specific role for both MAO-A and MAO-B was demonstrated for both reactions.

Sumatriptan is a serotonin 5-HT_1 receptor agonist used in the treatment of migraine and cluster headaches. The major clearance process was shown to involve oxidative *N*-deamination of the *N*-dimethyl side chain to form an indole acetic acid metabolite as shown in Fig. 3. Although both P450 and MAO may catalyze the oxidation of substituted amines such as this, Dixon et al. [14] showed that MAO-A is the major enzyme responsible for this transformation in human liver.

Propranolol, a β-adrenoreceptor blocker is used in the treatment of cardiovascular disorders and hypertension. It has been shown that the first step in propranolol metabolism is an *N*-dealkylation, followed by MAO-catalyzed deamination of the side chain (Fig. 4) [15].

2.1.2 Aldehyde Oxidase

Previously cited references [3, 4] have also reviewed this particular enzyme and the topic was recently covered in a very comprehensive fashion by Garattini, Fratelli, and Terao [16].

Aldehyde oxidases belong to the family of molybdo-flavoenzymes that require FAD and the molybdenum cofactor for activity. In contrast to MAO for example, the physiological role for AO is not well understood. As their name implies, aldehyde oxidases not only catalyze the oxidation of aldehydes to carboxylic acids, but they also catalyze the hydroxylation of heterocycles (typically aromatic *N*-containing systems). Comparison of the catalytic mechanisms between AO- and P450-mediated oxidations explains the crucial need for *N*-containing moieties in AO substrates. AO-catalyzed oxidations involve the nucleophilic addition of oxygen (water is the source) to an electrophilic site on the drug molecule such as *N*-containing heterocycles, with overall generation of reducing equivalents. In contrast, P450-catalyzed oxidations involve the formation of an electrophilic oxo species (molecular

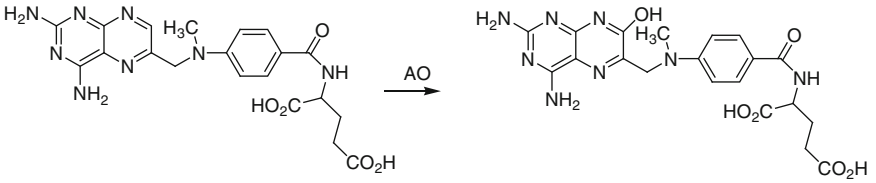
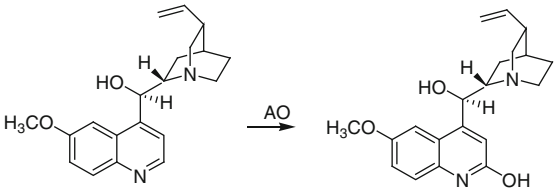


Fig. 5 AO-mediated aspect of methotrexate metabolism

Fig. 6 AO-mediated aspect of quinine metabolism



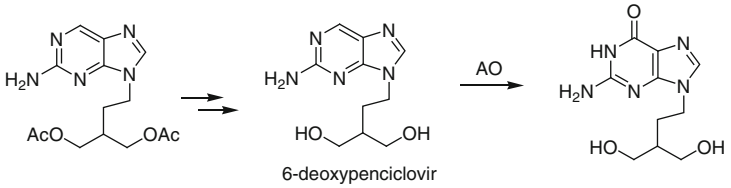


Fig. 7 AO-mediated aspect of famciclovir metabolism

oxygen is the source) which is then attacked by a nucleophilic site of the drug molecule, with overall consumption of reducing equivalents [3]. The substrate specificity for aldehyde oxidases is diverse and the list of drugs metabolized by AO spans a variety of pharmacological classes. To illustrate the structural diversity of potential AO substrates, representative examples of AO substrates shown are methotrexate, quinine, and famciclovir.

Methotrexate, a dihydrofolate reductase inhibitor, is used in the treatment of acute lymphocytic leukemia and rheumatoid arthritis. The hydroxylation at the 7-position is a major metabolic route catalyzed by AO (Fig. 5). Amongst the evidence confirming the role of AO in this important metabolic event [17, 18], it was observed that there is a high degree of inter-individual variability of methotrexate hydroxylation which correlates with AO levels [19].

Quinine is an antimalarial whose primary metabolic pathway appears to be oxidation catalyzed by aldehyde oxidase (Fig. 6) [20]. The 2′-quininone is the major metabolite formed.

Famciclovir, an antiviral agent, is a 9-substituted guanine derivative that undergoes bioactivation to yield the pharmacologically active moiety, penciclovir. Studies have shown that aldehyde oxidase catalyzes a key step in the bioactivation of this pro-drug (Fig. 7) [21, 22]. Famciclovir first undergoes hydrolysis followed by oxidation to yield

the active anti-herpes agent, penciclovir. Studies indicate that 6-deoxypenciclovir, the moiety penultimate to penciclovir, is acted upon by AO oxidation.

2.1.3 Flavin Mono-oxygenase

FMO is another enzyme for which myriad literature exists. Mitchell [23] has recently reviewed this enzyme and includes a particularly descriptive historical perspective of its discovery. FMO has been described as a complementary enzyme system to the P450s [24] because both are located in the endoplasmic reticulum (microsomes) and require NADPH and molecular oxygen for catalysis. In fact, at one time FMO-catalyzed reactions were believed to be simply another mixed function oxidase (P450) reaction. It was not until Ziegler and Mitchell [25] purified FMO that it was identified as distinct from P450. A detailed review of the similarities/differences between these systems has been described by Cashman [26], including the perspective that based on the properties of FMO, purposely designing molecules to incorporate FMO-mediated metabolism may actually offer a strategic advantage in drug design. Based on the sequence homology, FMOs have been grouped in families numbered 1–5. In human liver, FMO3 and 5 are the prominent species but in rat liver, FMO1 is predominant [26].

As noted, FMOs catalyze the *N*- or *S*-oxidation of heteroatom-containing compounds. Like the P450s, FMO-mediated oxidation is NADPH dependent; however, FMO operates via a two-electron mechanism while P450s operate via sequential one-electron processes. The suggestion has been made that this may actually contribute to the greater prevalence of P450 metabolism-based toxicity compared to FMO-related toxicity. To date, few physiological substrates for FMO have been identified so the details of known SAR are limited to "oxygenation of soft nucleophiles such as nitrogen or sulphur." The strategic advantage of incorporating FMO metabolism into synthetic design is based on the fact that few drugs used contemporarily are FMO substrates and therefore, new drugs metabolized by FMO should be less susceptible to drug–drug interactions with concomitant administration of other medications. Additionally, inter-individual variability of FMO appears to be due to genetic variation and not due to inhibition or induction. Thus, genetic screening may offer a simple way of predicting atypical drug metabolism. In a more nuanced approach to chemistry design based on application of FMO knowledge, it has been suggested that FMO-mediated oxidation coupled with retro-reduction will lead to futile metabolic pathways. There is in vitro evidence to support this phenomenon and literature data has suggested at least three prominent systems responsible for the retro-reduction of tertiary amine *N*-oxides [24]. It is conceivable that a perceived metabolic liability due to FMO-mediated metabolism may not be problematic if the forward metabolism is linked with a reverse retro-reduction. However intriguing this possibility may be, it is far from being established as a well-precedented approach.

As mentioned, compared to P450s, relatively fewer pharmaceutical agents to date have been reported to be FMO substrates. However, these have been recently catalogued by Phillips et al. [27]. The structural diversity of potential FMO

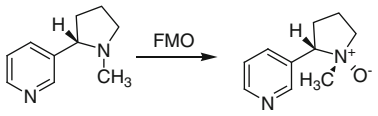


Fig. 8 FMO-mediated aspect of nicotine metabolism

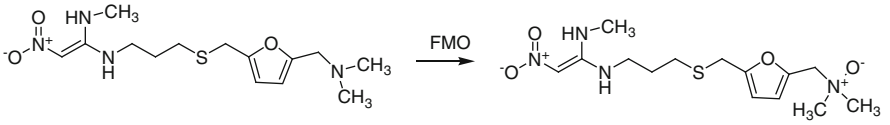


Fig. 9 FMO-mediated aspect of ranitidine metabolism

substrates can be illustrated with nicotine, ranitidine, and trimethylamine. Nicotine has been reported as a selective in vivo probe for FMO3 and the role of FMO in ranitidine metabolism has been described. The FMO-mediated *N*-oxidation of trimethylamine as a phenotyping marker for FMO has also been extensively reported on.

Nicotine is metabolized stereoselectively by FMO to form (*S*)-nicotine *N*-1′-oxide (Fig. 8). In a study involving thirteen healthy male smokers that examined free smoking, intravenous infusion of labeled (*S*)-nicotine-d_2 and dermal administration of (*S*)-nicotine-d_0, only the *trans* nicotine *N*-1′-oxide diastereomer was observed in the urine by all routes of administration [28]. This and other data point to the role of FMO3 in this stereoselective metabolism.

Ranitidine is an H_2 antagonist that has been used as an antiulcer drug that is metabolized almost exclusively to its *N*-oxide by FMO (Fig. 9) [29]. It has been shown in different populations that phenotypes of ranitidine *N*-oxidation are positively correlated with various FMO3 gene mutations [30, 31].

Finally, trimethylamine, derived from dietary choline, is metabolized by hepatic FMO3 to its non-odorous *N*-oxide metabolite. However, mutations in the FMO3 gene cause an inherited disorder known as trimethylaminuria (fish-odor syndrome) where affected individuals are unable to metabolize trimethylamine. The unchanged free amine is secreted through breath, sweat, and urine and because it possesses an odor reminiscent of rotting fish, such individuals face a significant social aversion to their condition, often leading to severe anxiety and depression. The condition itself is a phenotypic marker for FMO3 metabolism and studies have investigated the underlying genotypic basis for the disorder [27].

2.1.4 UDP-Glucuronosyltransferase

The UGT field is one of burgeoning research over the last several years, largely due to the investigation of the large number of isoforms in this enzyme family and particularly, research into the various polymorphisms associated with different

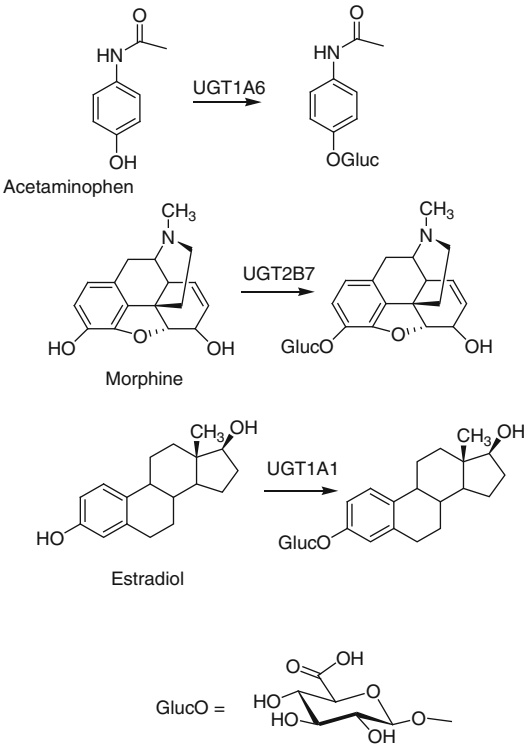


Fig. 10 UGT substrates illustrating structural diversity

isoforms. Two reviews, though well separated in time, give a comprehensive summary of this enzyme system [32, 33].

UGTs catalyze the conjugation of glucuronic acid to nucleophilic substrates. These may include phenols, aliphatic alcohols, carboxylic acids, and amines. There are a number of endogenous substrates for UGTs including bilirubin, various steroids, and lipids. All of which support the notion that UGT isoforms have broad and overlapping substrate specificities. Because glucuronidated metabolites significantly differ structurally from parent, glucuronidation generally renders metabolites that are pharmacologically inactive. However, there are exceptions and there is a body of literature describing the bioactivation and toxicity related to UGT-dependent metabolism [34]. Acetaminophen, morphine, and estradiol are glucuronidated by UGTs 1A6, 2B7, and 1A1 [35], respectively, illustrating the structural diversity accepted by this enzyme family (Fig. 10). While the P450-catalyzed bioactivation of acetaminophen has been well described, acetaminophen is also known to undergo direct glucuronidation catalyzed by UGT1A6. Morphine undergoes glucuronidation at both the 3- and the 6-positions, though morphine-3-glucuronide is quantitatively the more important of the two, with its formation being catalyzed by UGT2B7. While morphine-6-glucuronide (M6G) is thought to be a more potent analgesic than morphine itself, the role of morphine-3-glucuronide (M3G) is less clear. It has been hypothesized that M3G may actually antagonize the analgesic effects of morphine

and M6G and may also play a role in the development of tolerance [36]. The case of estradiol is interesting because UGT1A1 exhibited regioselectivity toward the 3-hydroxy position, whereas glucuronidation at the 17-hydroxy position was not detected. However, in a subtle SAR point, the rate of 3-hydroxy-glucuronidation was dependent on the 17-hydroxy configuration because 3-hydroxy glucuronidation for β-estradiol (shown) was six times faster than for epiestradiol [37].

It is clear that our knowledge and critically the predictive in vitro tools for studying these non-P450 drug metabolizing enzymes lag behind those for the P450s. Nevertheless, these enzymes can be important contributors to the metabolic clearance of compounds and it behooves the medicinal chemist to be aware of their properties. However, close collaboration with biotransformation scientists can facilitate this knowledge, thus leading to a better understanding of candidates and minimizing attrition due to poorly understood clearance mechanisms.

2.2 Reducing Metabolic Liability

Reducing metabolic liability is perhaps one of the most classical ways in which biotransformation can aid in chemistry design. It is also commonly referred to as "identifying metabolic hot spots" and traditionally has involved an iterative approach where the biotransformation scientist identifies specific sites of metabolism and chemists make appropriate substitutions based on these data to develop an SAR understanding of metabolic liability. The desired goal is that the SAR of metabolic liability can be optimized concurrently with the SAR required for pharmacological activity (and other parameters). The process is repeated until a compound with acceptable clearance can be identified. Of course, it will be recognized that changes in one parameter are generally not independent of others and it is likely the chemist will ultimately achieve an acceptable balance of properties rather than optimization of each aspect of the molecule [38]. The evolution of chemistry strategy to one of high speed analoguing raises the question as to whether this classical "metabolism blocking" approach is obsolete and can somehow be replaced by high throughput screening. For example, aromatic hydroxylation is a commonly observed type of metabolism, and furthermore it is often difficult for the biotransformation scientist to easily identify the exact position of the ring that is oxidized. However, a diversity of substituted aromatic rings are typically and readily available to explore the SAR of pharmacological activity. In this case, compared to a traditional metabolite identification study carried out by a biotransformation scientist on multiple compounds, in many cases it may indeed be faster for chemists to prepare analogues and look for changes in overall clearance rates via a high throughput assay that measures an endpoint such as clearance. However, rather than abandoning the traditional approach, we find that judicious timing of the metabolite identification studies can complement the screening assays and improve the overall efficiency of the process. For example, in the above-described situation, it might well be possible to simply prepare and screen many analogues; however, a

well-executed biotransformation study at the time of design can direct chemists to the best site for substitution and offer some insights as to other potential sites of metabolism, i.e., anticipating metabolic switching. Another advantage of these early studies is that they will inform as to whether the major site(s) of metabolism are also key elements required for pharmacological activity. In this case, when discriminating between different chemical series, it would clearly be preferable that the major clearance pathway not be associated with a moiety required for pharmacological activity. This could be used as an important way to differentiate between series.

Nevertheless, there is much interest in the prediction of metabolites and their accurate prediction at early stages such as lead selection would represent a significant way to accelerate this stage of discovery. To that end, the in silico prediction of metabolites has been an active field of investigation; however, a review of the various approaches or the evaluation of specific programs is beyond the scope of this chapter. The reader is directed to several reviews for further information [39–41]. Czodrowski et al. [39] have nicely summarized the current state and concluded that "while considerable progress has been made, the results of calculations still need careful inspection. . .and the domain of applicability as well as methodological limitations have to be taken into account." It is generally acknowledged that metabolism is one of the more difficult absorption, distribution, metabolism and excretion (ADME) endpoints to predict because of its complexity – competing mechanisms and enzymes, inherent reactivity of the substrate, ligand–protein interactions, genetic and phenotypic variations, all of which are factors that contribute to the metabolic process.

The following are illustrative examples of the traditional experimental approach that have positively impacted SAR development and illustrate various aspects of the approach: (1) metabolic blocking, (2) purposely diverting/shunting metabolism, (3) indirect modulation of metabolism by F substitution, and (4) metabolic switching.

2.2.1 Metabolic Blocking

Methotrexate is an antifolate used in the treatment of rheumatoid arthritis. The aldehyde oxidase liability associated with methotrexate was previously described. However, the complete metabolism picture for methotrexate is quite complex, impacting its pharmacodynamic action and making its pharmacokinetics less predictable [42]. In addition to AO, methotrexate may be acted upon by folylpolyglutamate synthetase and carboxypeptidase G2. The AO-catalyzed formation of the 7-hydroxy metabolite of methotrexate diminishes its efficacy because the metabolite is less soluble and much less potent at the desired target than the parent compound itself. Poly(γ-glutamyl) metabolites of methotrexate are the primary contributors to long-term methotrexate retention but their role in methotrexate efficacy and toxicity is controversial. Finally, methotrexate is known to undergo enterohepatic recycling, i.e., secretion into bile followed by reabsorption in the intestine. Metabolism by carboxypeptidase G-like activity will likewise diminish

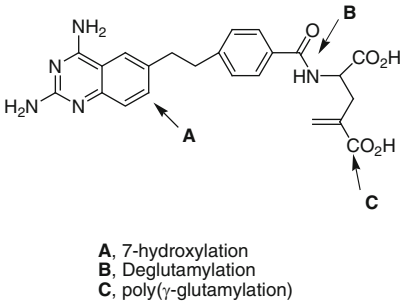


Fig. 11 Sites and drug metabolizing enzymes targeted for metabolic blocking

methotrexate efficacy. Clearly, antifolates that do not undergo these types of metabolism would be superior to methotrexate. CH-1504 was designed to be "metabolism-blocked" for all three metabolic pathways (7-hydroxylation, polyglutamylation, and deglutamylation) [43]. In a small, nonblinded study of RA patients, CH-1504 was superior to methotrexate in efficacy and tolerability. Encouraged by this success, investigators have designed analogues with even greater potential therapeutic efficacy [42]. CH-1504 is shown in Fig. 11 with the three problematic types of metabolism indicated. All analogues prepared contained the 4′-methylene-Glu and examined the effects of various other substitutions. The 5,8-dideaza-pyrazine ring of CH-1504 and its analogues was key to mitigating AO activity. In an assay using rabbit liver AO, methotrexate showed a 2 min half-life while none of the analogues examined showed any propensity to act as substrates for this enzyme. All analogues (except one) were inert to cleavage by poly (γ-glutamyl) synthetase activity. Finally, carboxypeptidase G2 activity from *Pseudomonas* sp. strain RS-216 has been shown to catalyze the terminal amino acid hydrolysis from methotrexate. Under similar experimental conditions, no hydrolysis of any of the analogues occurred showing that they are not substrates for this enzyme. Compared to methotrexate, the key to the metabolic stability of these analogues is incorporation of the 4′-methylene-Glu in place of Glu and removal of the nitrogens to give the 5,8-dideaza-pyrazine ring system.

Another example of the metabolic blocking approach can be exemplified to minimize P450-catalyzed metabolism. Extensive and intensive research through the 1990s led to the identification of human immunodeficiency virus (HIV) protease inhibitors for the treatment of (HIV-1) infections. However, this class of agents, exemplified by indinavir, generally suffered from extensive first-pass metabolism. Attempts to develop agents with improved pharmacokinetic properties focused on blocking the sites of metabolism (typically mediated by P4503A4). Cheng et al. [44] deconstructed the metabolic liabilities of indinavir and designed in attributes that significantly enhanced their PK properties. Indinavir undergoes metabolism at two regions of the molecule designated as P_2' and P_3 as shown in Fig. 12. Specifically, oxidation takes place at the benzylic position of the aminoindanol moiety (P_2'), in addition to the pyridine nitrogen and the methylene linker (P_3). Incorporation of the gem-dimethyl and pyridylfuran functionalities in P_3 (compound **1**), along with replacement of the aminoindanol with an aminochromanol moiety (compound

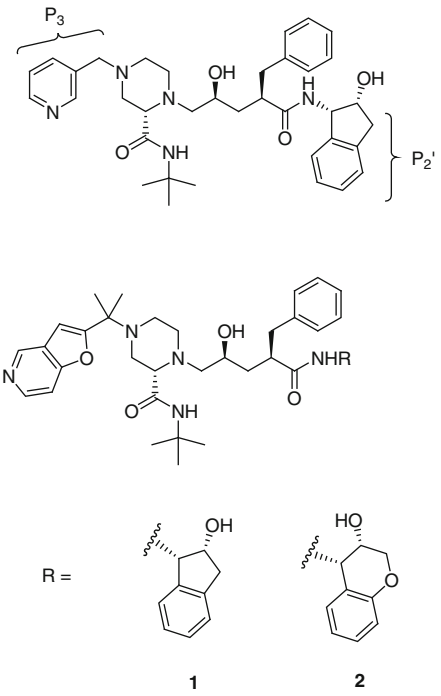
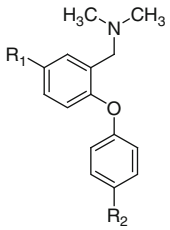


Fig. 12 Indinavir and analogues designed based on metabolic blocking

2) led to analogues as shown in Fig. 12. In two markers of pharmacological activity [(1) inhibition of cleavage of a substrate by the wild-type HIV-1 protease enzyme and (2) inhibition of the spread of viral infection in MT4 human T-lymphoid cells infected by the NL4-3 virus], both compounds showed enhanced potency compared to indinavir. The pharmacokinetic profiles of these two molecules were then examined in dogs. When corrected for dose, compound **1** showed twofold improvement in C_{max} and AUC, while compound **2** showed more marginal improvement (1.2–1.5-fold, respectively).

2.2.2 Purposely Diverting/Shunting Metabolism

In a slightly different approach, rather than designing out a particular metabolic hot spot, Middleton et al. report on purposely redirecting metabolism away from an undesired site [45]. In a program designed to identify selective serotonin reuptake inhibitors, these investigators identified compound **3** as a promising lead (Fig. 13). Unfortunately, balanced with a number of desired attributes, the molecule was found to undergo P4502D6-mediated *N*-demethylation and the resulting secondary amine was both pharmacologically active and possessed a relatively long half-life, which was inconsistent with the clinical objectives of this program. Previous experience suggested that P450-mediated thio alkyl *S*-oxidation can be a rapid metabolic process



3, $R_1 = NHSO_2Me$; $R_2 = CF_3$
4, $R_1 = F$; $R_2 = SMe$

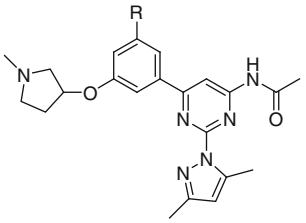
5, $R_1 = SO_2NH_2$; $R_2 = SMe$
6, $R_1 = SO_2NH_2$; $R_2 = S(O)Me$

Fig. 13 Modifications to enable metabolic shunting

and might be an attractive competing event to *N*-demethylation. Indeed, a simple thio methyl analogue **4** illustrated the concept. Based on the analysis of substrate docking using homology models and a further expansion of the SAR, the team arrived at sulfonamide **5**. This compound was potent and selective for serotonin over dopamine and noradrenaline reuptake inhibition. In vitro, the sulfoxide **6** was the predominant metabolite (>90%) and showed only weak pharmacological activity ($IC_{50} > 1$ μM). In vivo, in both rat and dog pharmacokinetic studies, the parent compound **5** retained the desired ADME properties and indeed as predicted by the in vitro studies, the sulfoxide **6** was the predominant metabolite. It possessed the desired short half-life and furthermore was shown to be inactive against a broad panel of other receptors, enzymes, and ion channels. Based on the cumulative knowledge, sulfonamide **5** was progressed into clinical development.

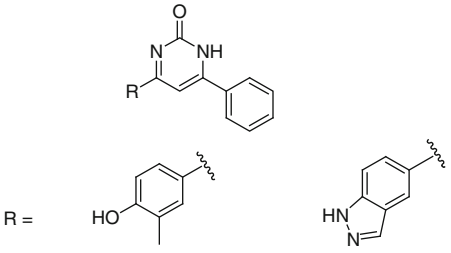
2.2.3 Indirect Modulation of Metabolism by F Substitution

Selective A_{2a} antagonists are of interest for the treatment of Parkinson's disease. Moorjani et al. [46] reported on the use of fluorine substitution to enhance metabolic stability in a series of non-xanthine-based antagonists with a pyrimidine core as shown in compound **7** (Fig. 14). The compound shown possessed good potency and selectivity; however, the projected human intrinsic clearance was 54 mL/min/kg. SAR had shown that the aromatic ring would tolerate various fluorine substitutions and indeed, compound **8** showed single digit nanomolar potency for hA_{2A} with 135-fold selectivity over hA_1. Importantly, the projected human intrinsic clearance was reduced to 3 mL/min/kg, presumably because the electron-withdrawing effects of the fluorine stabilized the phenyl group to metabolism. The effects of fluorine on all ADME properties, including metabolism, have been known and exploited by medicinal chemists for some time [47]. The nature of the stereoelectronic effects, bond lengths and strengths, etc. has been well described. However, the understanding of the influence of fluorine substitution on docking interactions, whether through



7, R = H
8, R = F

Fig. 14 Modulation of metabolism by fluorine substitution



9 **10**

Fig. 15 Attempted metabolic blocking by isosteric replacement

direct contact with proteins or via drug conformation conferred via stereoelectronic effects, is more nascent but has been recently reviewed [48].

2.2.4 Metabolic Switching

The serine/threonine kinase CDC7 is involved in initiation of DNA replication in eukaryotic cells and has emerged as an attractive target for cancer therapy. Shafer et al. [49] have reported on pyrimidin-2(1*H*)-one compounds that emerged from a high throughput screen and are potent inhibitors of CDC7. However, compounds exemplified by **9** (Fig. 15) showed a high metabolic liability with glucuronidation of the phenol as the major metabolic pathway. Quantum mechanical conformational analysis suggested that an indazole (compound **10**) would be a suitable isostere for the phenol, thus blocking metabolism. Compound **10** was prepared and was approximately threefold less potent than **9**; however, further elaboration of the distal aromatic ring (chlorination at the 3-position) achieved compounds with the desired potency (0.005 μM). Unfortunately, compound **10** showed no improvement in metabolic stability. This example highlights one of the cautions in the metabolite blocking approach – metabolic enzymes can be extremely promiscuous and substitutions in one part of the molecule may simply lead to metabolism somewhere else, i.e., metabolic switching.

It seems clear to us that traditional metabolic hot spot biotransformation studies still have relevance to current chemistry approaches that rely on high speed analoguing. The value of the mechanistic understanding these studies provide has

been demonstrated to illustrate different aspects of their application. While these studies are not typically scalable for high throughput use, more importantly, they are customizable to address the question/problem at hand and can have tremendous impact on chemistry design.

2.3 Reactive Metabolites

Idiosyncratic adverse drug reactions (IADRs) (also known as type B ADRs) are a safety concern [50] whose study generally falls under the purvey of biotransformation scientists. These ADRs are often life threatening and are especially problematic because they are not attributable to any specific pharmacology, are typically unrelated to the drug target, and are generally dose independent. Their incidence is rare (1 in 10,000 to 1 in 100,000) and animal toxicity studies have poorly predicted the finding in humans [51]. Because of these factors, and the fact that they occur relatively late in development, many pharmaceutical companies are trying to identify these potential safety liabilities earlier in the drug discovery process. Understanding a molecule's potential to form reactive metabolites is one aspect of this approach.

2.3.1 Metabolism-Based Toxicity

Generally, metabolism of a drug results in a more polar species that is more readily excreted, with no toxicological sequela. However, for some structural moieties, metabolism can generate electrophilic, reactive metabolites (bioactivation). When the capacity of natural detoxification mechanisms for reactive metabolites is exceeded, toxicity may ensue (e.g., tissue necrosis, carcinogenicity, teratogenicity, and/or certain immune-mediated idiosyncratic toxicities).

The notion of linking metabolism-based bioactivation with toxicity extends back decades to studies demonstrating the bioactivation of aminoazo dyes into protein reactive metabolites [52]. However, studies with the anti-inflammatory agent acetaminophen [53–56] really laid the groundwork for this concept in drug metabolism. In short, acetaminophen undergoes P450-mediated bioactivation to a reactive quinone-imine metabolite (NAPQI) [57]. This reactive intermediate is capable of depleting levels of the endogenous anti-oxidant glutathione (GSH) and binding covalently to liver macromolecules thus leading to toxicity. Understanding these events and the various experimental approaches developed from them have guided biotransformation studies since then.

2.3.2 Are Reactive Metabolites Toxic?

Despite decades of research, the covalent binding of reactive drug metabolites to proteins as it relates to IADRs remains poorly understood. Generally, acetaminophen

being an exception, there is a lack of predictive animal models for IADRs which is a tremendous burden to drug development. Several hypotheses for IADRs exist, but one that links the formation of reactive metabolites with IADRs (especially those with a possible immune component) is the immune hypothesis. In this case, reactive metabolites can bind to high molecular weight proteins creating neoantigens and initiating an immune cascade, such as with penicillin-induced anaphylactic reactions [58], or β-lactam- and sulfamethoxazole-induced skin rash [59]. Additional examples of drugs associated with haptenization include halothane, tienilic acid, and dihydralazine, all of which are bioactivated to reactive metabolites and display mechanism-based inactivation of the P450 isozymes responsible for their metabolism. Consistent with these observations, antibodies detected in sera of patients exposed to these drugs specifically recognize P450 isozymes responsible for their metabolism [60–62].

2.3.3 Reactive Metabolite Screening

Under the assumption that reactive metabolites are a necessary, though not sufficient step in certain IADRs, most pharmaceutical companies have implemented assays to evaluate the bioactivation potential of new compounds through some type of characterization of reactive metabolites.

Reactive Metabolite Trapping

Reactive metabolites are generally identified indirectly by trapping and forming a stable conjugate such as with GSH. The presence of the soft nucleophilic thiol group in GSH or its corresponding ethyl ester ensures efficient conjugation to soft electrophilic centers on some reactive metabolites (e.g., Michael acceptors, epoxides, arene oxides, and alkyl halides) yielding stable sulfydryl conjugates [63–66]. As described previously, drug metabolizing enzymes other than P450 are also capable of catalyzing bioactivation, and therefore the choice of in vitro metabolism system (e.g., liver cytosol, liver S-9 fractions, hepatocytes, and neutrophils) must reflect the activity of the appropriate enzymes. Analytically, loss of the pyroglutamate group from the GSH conjugates can be followed by mass spectrometry (constant neutral loss of 129 Da) to provide relatively sensitive detection [67]. In contrast to soft electrophiles, hard electrophiles (e.g., electrophilic carbonyl compounds) will preferentially react with hard nucleophiles such as amines (e.g., semicarbazide and methoxylamine), amino acids (e.g., lysine), and DNA bases (e.g., guanine and cytosine) affording the corresponding Schiff base [68, 69]. Cyanide anion is another "hard" nucleophile that can be used to trap electrophilic iminium species that are generated via metabolism of acyclic and cyclic tertiary amines [70, 71].

Acyl glucuronides (derived from the glucuronidation of carboxylic acids) represent a special case of reactive metabolites because they are relatively long-lived. Their propensity for toxicity has been correlated with their chemical stability as

assessed by comparison of pseudo first-order degradation half-life values of the 1-*O*-acyl glucuronides and rate of acyl migration in in vitro studies [72, 73].

Covalent Binding

As stated, formation of reactive metabolites is a necessary, though not sufficient first step in the pathogenesis of certain IADRs. The next committed step in these cases is covalent binding of the reactive metabolite to some protein and therefore, quantitative assessment of the amount of in vitro metabolism-dependent covalent binding to biological tissue has been explored [74]. However, such studies are only possible if radiolabeled drug is available, but importantly, the assay does not reveal the nature or identity of covalently modified proteins. Such covalent studies may also be performed in vivo, but study design must be carefully considered. For example, toxicity with acetaminophen is only seen upon multiple dosing when the naturally efficient detoxification mechanism (conjugation of NAPQI with GSH) is overcome [55].

2.3.4 Structural Alerts

As described, a metabolic transformation leading to bioactivation (vs. a metabolic transformation leading to a benign elimination) depends on the presence of a certain functionality (referred to as structural alert/toxicophore). Based on the precedented examples of drugs containing moieties which are metabolized to reactive metabolites and are associated with IADRs [64, 65], certain functional groups have been categorized as structural alerts. Clearly, avoiding these functional groups when possible is desirable; however, novel or unanticipated bioactivation pathways leading to reactive metabolites are always possible. This is illustrated for the 5-hydroxytryptamine 5-HT_{2C} agonist and potential anti-obesity agent 2-(3-chlorobenzyloxy)-6-(piperazin-1-yl)pyrazine (**11**) (Fig. 16) [75] which was discontinued from clinical development due to S-9/NADPH-dependent genotoxic effects in the bacterial *Salmonella* Ames assay. Subsequent reactive metabolite trapping studies in S-9/NADPH incubations containing exogenously added hard and soft nucleophilic trapping agents led to the identification of several conjugates of **11** and its downstream metabolites, revealing bioactivation on both the 3-chlorobenzyloxy and the piperazine ring system in **11** (Fig. 16). Bioactivation of the piperazine ring system was especially interesting given its wide usage and safety profile in commercially successful drugs like sildenafil.

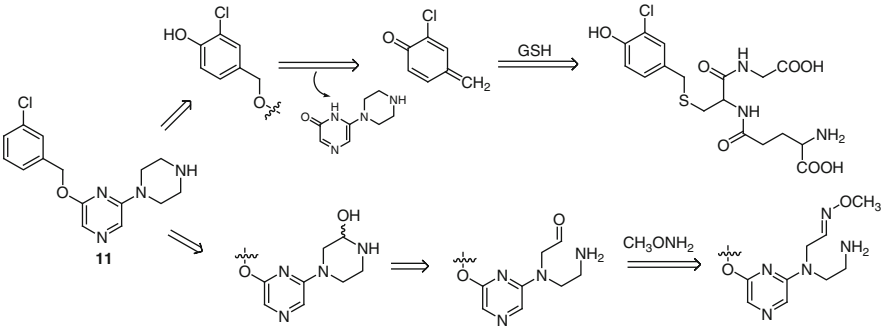


Fig. 16 Proposed mechanism of bioactivation of the 5-HT_{2C} agonist **11**, which leads to the formation of DNA-reactive metabolites

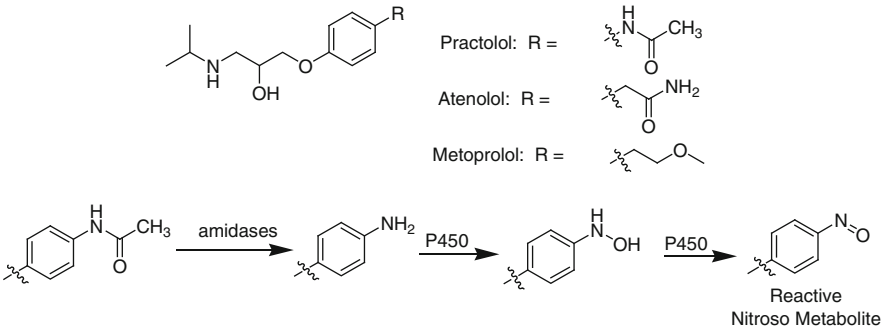


Fig. 17 Structure–toxicity relationships with β-adrenoceptor antagonists practolol, atenolol, and metoprolol

2.3.5 Does Avoiding Structural Alerts Guarantee Successful Drug Design?

If structural alerts/toxicophores are susceptible to bioactivation, it follows that drugs which lack toxicophores will have a superior safety record with regards to IADRs and anecdotal evidence supports this [64]. Examples where the metabolism data supports this hypothesis can be illustrated with the cardioselective β-adrenoceptor antagonists practolol, atenolol, and metoprolol. The mechanism of severe skin rashes induced by practolol is uncertain; however, a role for antinuclear antibodies, elicited by protein adducts of a reactive nitroso metabolite obtained from practolol biotransformation (liberation of a masked aniline) has been suspected (Fig. 17) [76, 77]. Notably atenolol and metoprolol are metabolized by completely different pathways and are also subject to extensive urinary excretion as parent drugs [78]. Both lack the anilide toxicophore and cutaneous IADRs are not observed with these drugs.

As a final example, bioactivation of the alkylhalide substituents of inhaled anesthetics to reactive acylating agents is usually due to the availability of an

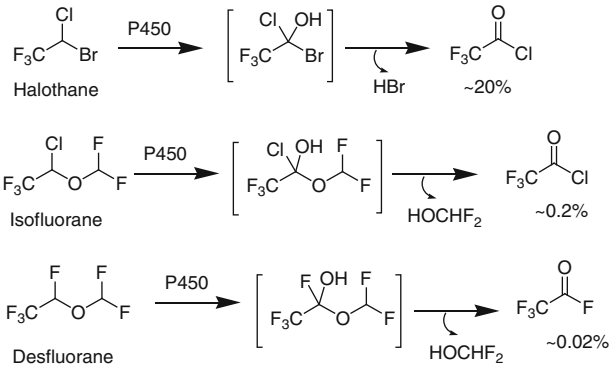


Fig. 18 Varying degrees of anesthetic metabolism

extractable hydrogen atom on the halogenated alkyl carbon. In susceptible patients, halothane, isoflurane, and desflurane can produce severe hepatic injury by an immune response directed against reactive acyl halides covalently bound to hepatic biomacromolecules [79]. The relative incidence of hepatotoxicity due to these agents appears to directly correlate with the extent of their conversion to acyl halides by P450, which in turn may be governed by the leaving group ability of the respective substituents within these drugs. As is seen in Fig. 18, halothane, which exhibits the greatest incidence of hepatotoxicity in the clinic, undergoes the most conversion to reactive acyl chloride, a feature that can be attributed to the presence of bromide substituent, which is a good leaving group. In contrast, isofluorane and desfluorane also undergo oxidative metabolism resulting in the formation of reactive acyl halides, but the degree to which these anesthetics are bioactivated is significantly lower than halothane [80]. Thus, the lower yield of acylhalide formation with isofluorane may be traced back to changes in the electronic environment that reduce overall affinity towards metabolism or to the relatively poor leaving group ability of the difluoromethoxy group compared to the bromide.

Given the lack of methodology to predict IADRs, the examples discussed above suggest that by avoiding toxicophores in drug design, one would lessen the odds that a drug candidate will lead to toxicity via a bioactivation mechanism (metabolism-based toxicity). However, avoiding structural alerts altogether can lead to missing out on potentially important medicines, as illustrated with atorvastatin, which not only contains the acetanilide structural alert, but metabolism by P450 results in the formation of acetaminophen-like metabolites (Fig. 19) [81]. Furthermore, glucuronidation of its carboxylic acid moiety results in the formation of the potentially electrophilic acyl glucuronide [82] in a manner similar to that discerned with certain nonsteroidal anti-inflammatory drugs. The toxicity associated with these carboxylic acid-based anti-inflammatory drugs has been attributed to the rearrangement of the acylglucuronide metabolites [72, 73].

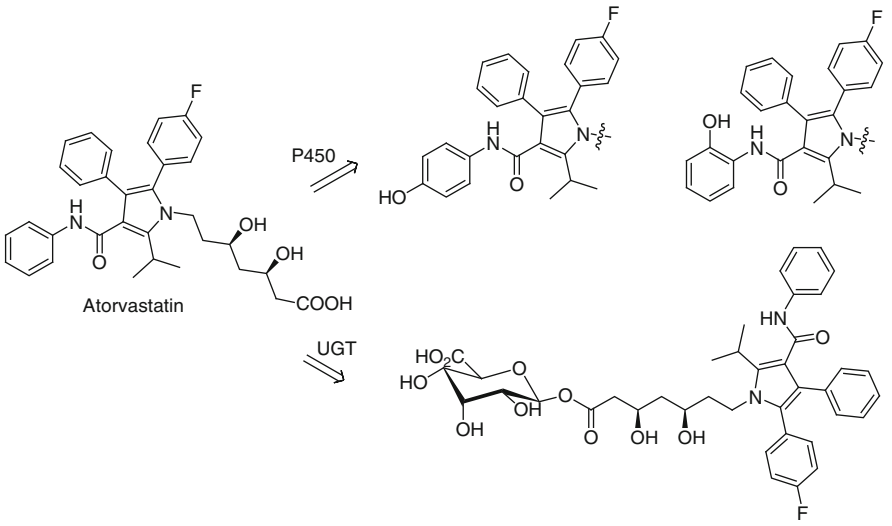


Fig. 19 Chemical structures of atorvastatin and its metabolites derived from oxidative and conjugation pathways

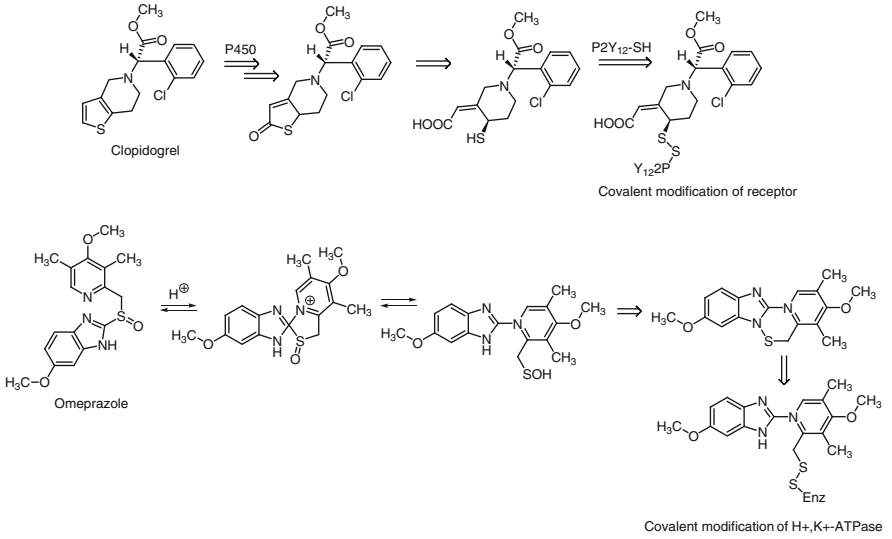


Fig. 20 Examples of commercial blockbuster drugs, which require reactive metabolite formation for their pharmacologic action

Other examples where bioactivation occurs with no apparent associated toxicity are clopidogrel [83–85] and omeprazole [86, 87] (Fig. 20). In fact, these agents actually depend upon bioactivation as part of their mode of action.

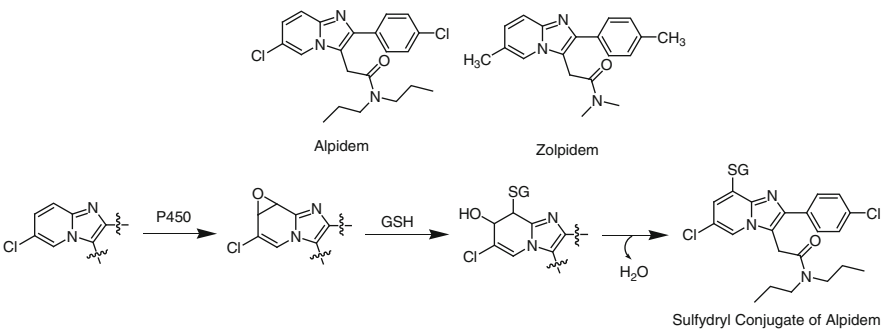


Fig. 21 Differential metabolism of the anxiolytic agents, alpidem (hepatotoxin) and zolpidem (non-hepatotoxin)

While avoiding toxicophores is generally a prudent chemistry strategy, there are sufficient exceptions (e.g., atorvastatin, clopidogrel, and omeprazole) that confound implementing such a simple, one-size-fits-all approach. Because reactive metabolite formation is a necessary, but not sufficient event potentially leading to toxicity, it is important to consider other factors that will also influence the entire toxicological pathway for compounds containing a structural alert. These factors include: (1) availability and relative contribution of alternate metabolic pathways that compete with structural alert bioactivation and (2) the existence of metabolic/detoxification pathways that efficiently scavenge the reactive metabolite and/or its precursor. Comparison of the bioactivation potential of the benzodiazepine receptor ligands, alpidem and zolpidem, exemplifies the first point. Alpidem is hepatotoxic and has been withdrawn from the market while zolpidem is devoid of the toxicity and is commercially successful. A key structural difference in the two drugs is the replacement of the two chlorine atoms on the imidazopyridine nucleus in alpidem with two methyl groups in zolpidem. In alpidem, the imidazopyridine ring is bioactivated by P450 leading to the formation of a reactive arene oxide that reacts with GSH to yield sulfydryl conjugates (Fig. 21), which have been detected in human excreta [88]. While bioactivation via epoxidation is also likely in zolpidem, the molecule does not undergo this metabolic fate; instead the two methyl groups function as metabolic soft spots and are oxidized to the corresponding alcohol and carboxylic acid metabolites.

The selective estrogen receptor modulator raloxifene exemplifies the importance of detoxication pathways. Raloxifene undergoes in vitro P4503A4-catalyzed bioactivation on its phenolic groups to yield reactive quinonoid species (Fig. 22) [89]; however, in vivo, glucuronidation of the same phenolic groups in the gut and liver constitutes the principal elimination mechanism of raloxifene in humans [90]. Thus, the likelihood of raloxifene bioactivation in vivo is in question when compared with the phase II glucuronidation process, a phenomenon that may provide an explanation for the extremely rare occurrence of IADRs.

Much attention has been focused on the use of covalent binding in predicting IADRs [74] but only recently was its predictive validity for idiosyncratic

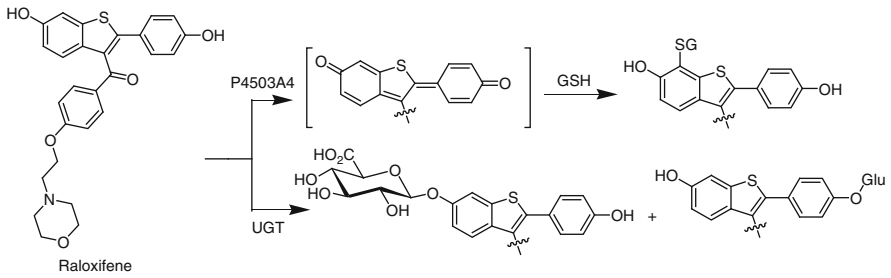


Fig. 22 Bioactivation and competing detoxication pathways of the selective estrogen receptor modulator, raloxifene

hepatotoxicity specifically tested. Obach et al. [91, 92] examined the binding of 18 drugs (nine hepatotoxins and nine non-hepatotoxins) to human hepatic tissue, taking into consideration key factors such as those outlined above, i.e., alternate metabolic pathways and detoxification pathways. Unsurprisingly, most of the hepatotoxic drugs demonstrated covalent binding to some degree, but surprisingly, several non-hepatotoxic, commercially successful drugs, also demonstrated covalent binding (e.g., buspirone, diphenhydramine, meloxicam, paroxetine, propranolol, raloxifene, and simvastatin). Detailed analysis of the data to discriminate between hepatotoxic and non-hepatotoxic drugs revealed no measure by which some drugs would ultimately not be miscategorized.

2.3.6 Lower the Dose

Perhaps the single most important factor in mitigating IADR risks appears to be the daily dose of the drug. There are no examples of drugs (even those that undergo bioactivation) that are dosed at $<$20 mg/day that cause IADRs. For two drugs with identical structural alerts susceptible to bioactivation, the one administered at the lower dose is typically safer than the one given at a higher dose. For example, the dibenzodiazepine derivative olanzapine (Fig. 23) forms a reactive iminium metabolite very similar to the one observed with clozapine, yet olanzapine is not associated with a significant incidence of agranulocytosis. This is likely due to a marked reduction in the total body burden to reactive metabolite exposure which is therefore unlikely to exceed the threshold needed for toxicity. Clozapine is given at a dose of $>$300 mg/day, while the maximum recommended daily dose of olanzapine is 10 mg/day. Tadalafil and the anti-hypertensive drug, prazosin, similarly illustrate the point (see Fig. 23). The methylenedioxyphenyl group in tadalafil undergoes P4503A4-catalyzed bioactivation to an electrophilic catechol, a process that also leads to the suicide inactivation of P4503A4 activity in vitro [93]. However, to date there are no reports of IADRs or P4503A4 drug–drug interactions associated with tadalafil use at the recommended dose of 10–20 mg/day. Likewise, there are no reports of IADRs with prazosin at the recommended daily dose of 1 mg/day, despite the bioactivation of the pendant furan ring to electrophilic

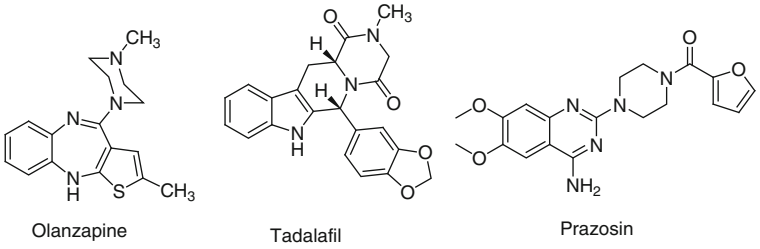


Fig. 23 Examples of low daily dose drugs devoid of IADRs despite bioactivation liability

intermediates, trapped with GSH and semicarbazide [94]. Additional evidence to support the dose/idiosyncratic toxicity hypothesis has emerged from recent studies on the covalent binding of 42 drugs (categorized into safe, warning, black box warning, and withdrawn) to human hepatic tissue and rat liver in vivo [95]. Consistent with the observations of Obach et al. [91, 92], straightforward covalent binding assessments in human hepatic tissue and/or rat liver following oral administration of the test compound did not distinguish the safety categories. However, regression analysis of the log-normalized covalent binding versus log-normalized daily dose data suggested a relationship between covalent binding, daily dose, and toxicity [95].

2.3.7 Concluding Remarks

Decades of research have failed to yield an unequivocal chemistry strategy to deal with reactive metabolites. Reactive metabolite formation in a chemical series should also consider alternate metabolic pathways, competing detoxification pathways, and an estimation of the human dose based on pharmacokinetic/pharmacodynamic studies in preclinical species. Bioactivation is only one aspect of the overall risk/benefit assessment for advancing a drug candidate into development.

IADRs are complex and currently we lack sufficient mechanistic understanding to be able to replicate them experimentally. Similarly, this lack of understanding means no predictive in vivo models exist. Genetic factors appear to have a crucial role in the induction of IADRs and therefore, future approaches may lie in this area. For instance, retrospective single nucleotide polymorphism analysis of a population (500,000) exposed to the HIV agent abacavir suggested that the known HLA-B gene region could be a predictive genetic marker for a hypersensitivity reaction. Further analysis showed the risk could be identified with as few as 15 cases and 200 population controls and has now been instituted in practice to avoid the side effects [96]. An additional area of research includes studies on the identities of the protein targets of reactive metabolites discerned with toxic versus nontoxic drugs and on the combined application of covalent binding measurements with transcriptomic, metabonomic, and proteomic technologies in an effort to discern (and thereby predict) the characteristics of a toxic response. Until we develop a better

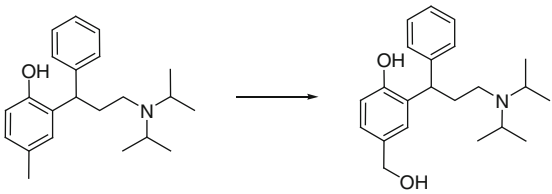


Fig. 24 Structure of tolterodine and its active hydroxymethyl metabolite

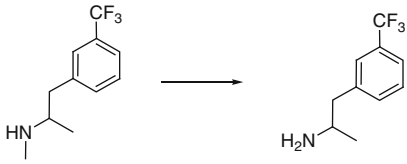


Fig. 25 Structure of fenfluramine and its active *N*-desmethyl metabolite

understanding of the risk of toxicity arising from the formation of reactive metabolites, the advancement of potent (low dose) drug candidates with only a limited propensity to form reactive intermediates would appear to be the most favored strategy.

2.4 Active Metabolites

2.4.1 Introduction to the Importance of Metabolite Activity

In the course of metabolite identification studies during both drug discovery and drug development, much consideration is given to the pharmacological activity of metabolites, due to its potential significance on efficacy and safety. Indeed, numerous examples exist where a metabolite of the administered compound is the key active component, as observed for the antimuscarinic receptor antagonist tolterodine [97], where the 5-hydroymethyl metabolite has similar pharmacological activity and contributes significantly to the clinical efficacy (Fig. 24) [98].

In addition, active metabolites may give rise to deleterious side effects, resulting in compound attrition or, if not detected early enough, compound withdrawal, as was the case for the 5-hydroxytryptamine receptor agonist fenfluramine, which formed an *N*-dealkylated metabolite in humans of greater potency than the parent compound against not only the target $5HT_{2C}$ receptor but also the $5HT_{2B}$ receptor, resulting in hypertension and valvular heart disease (Fig. 25) [99–101].

The numerous examples of this type that have been observed over the years have led to an increased interest in the characterization of metabolite activity during drug discovery, either to pre-empt issues that may result in compound attrition or indeed to design improved molecules as a result of a deeper knowledge of metabolite pharmacology.

2.4.2 Approaches to Characterize Metabolite Activity

Historically, metabolite activity characterization during drug discovery has involved metabolite identification studies, using either standard in vitro approaches (involving metabolizing systems such as microsomal or hepatocyte preparations) or in vivo samples from animal species, followed by chemical synthesis and pharmacological testing of the potentially active structure. While in principle, all identified metabolites can be synthesized and tested for activity, in practice relatively few metabolites are chosen for testing, due to the significant resources that may be required for the chemical synthesis, depending on the structure of the metabolite. The choice of metabolites to be tested is commonly based on the specific structure of the metabolite and knowledge of the pharmacophore of the parent molecule: notably, it is well established that metabolites of close structural resemblance to the parent compound are more likely to show activity against the target, while those with significantly different structures are far less likely to do so. With the increased need for information on the pharmacological activity of metabolites earlier in the discovery phase; however, alternative approaches to yield activity data have been developed. A simple approach to assess the presence of active metabolites is to profile the samples by HPLC and then submit the collected fractions for activity testing. Although not amenable to the quantitative assessment of activity, such an approach can identify the presence of previously undetected active metabolites in in vitro or in vivo samples and is far less resource intensive than chemical synthesis. The discovery of a component with potentially significant activity can then drive further work to assess the potency of the metabolite in question. A more detailed methodology for the assessment of active metabolites that has been developed in recent years involves online profiling of metabolite samples [102–105]. Such approaches, using analytical systems that potentially involve the integrated formation, chromatographic separation, quantitation, identification, and pharmacological testing of metabolites, enable high-throughput metabolite activity assessments and are thus highly suitable for the early discovery arena.

2.4.3 Drug Design via Pharmacological Activity Profiling

The ability to profile metabolite activity during drug discovery offers the potential to significantly impact the design of novel drugs. Notably, activity profiling can lead directly to the discovery of compounds with improved drug characteristics, including pharmacological properties, pharmacokinetic parameters, and overall safety profile. In addition, the re-design of chemical series to more suitable chemical matter in an appropriate time frame can be achieved using this approach. A classic example of an active metabolite with improved characteristics over its parent compound is the antihistamine agent fexofenadine, a carboxylic acid metabolite of the marketed product terfenadine (Fig. 26) [106]. The biotransformation of terfenadine to fexofenadine is predominantly mediated by the P4503A4 enzyme and results in

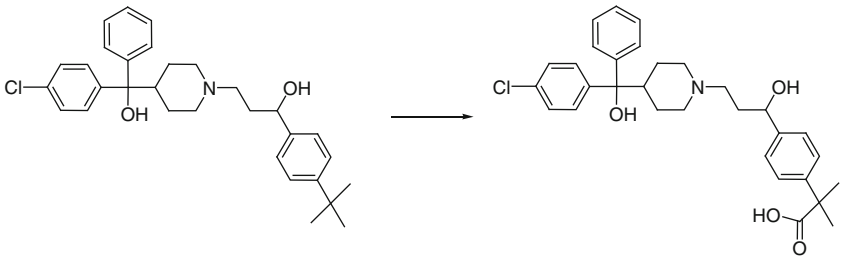


Fig. 26 Structures of the antihistamine agents terfenadine and its metabolite fexofenadine

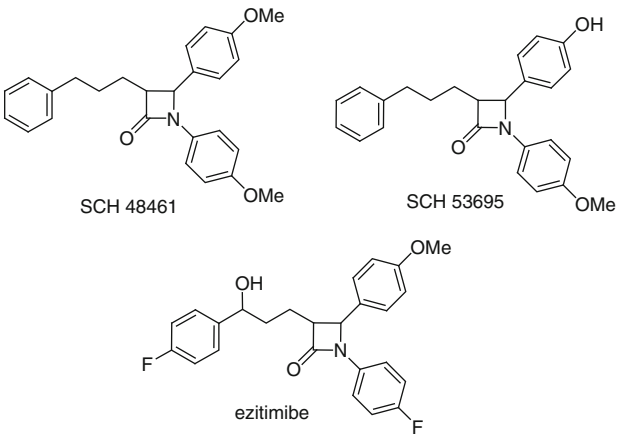


Fig. 27 Structure of the prototype cholesterol absorption inhibitor SCH 48461, its *O*-desmethyl metabolite, SCH 53695 and the final product ezitimibe

significant first-pass metabolism of terfenadine in humans. Terfenadine itself is now known to be a potassium channel inhibitor, leading to QTc interval prolongation, such that co-administration of terfenadine with a P4503A4 inhibitor results in potentially fatal cardiac arrhythmias [107]. When terfenadine was withdrawn from the market as a result of these clinical observations, it was possible to replace the compound with fexofenadine, which does not cause cardiac issues.

While the discovery of fexofenadine was as a result of clinical observation rather than rational chemical design, an excellent example of drug discovery via an understanding of the activity profile of drug metabolites has been described for the cholesterol absorption inhibitor ezitimibe (Fig. 27) [108, 109]. The prototype compound of the chemical series (SCH48461) was shown to have good activity in animal models, despite being rapidly and extensively metabolized, implicating the formation of active products of biotransformation. Using a series of in vivo studies, a potent metabolite of the lead compound was discovered, produced via *O*-demethylation and subsequent glucuronidation of the resulting phenol. The learnings from this discovery

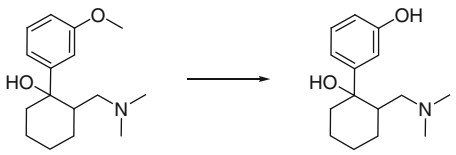


Fig. 28 Structure of tramadol and its active *O*-desmethyl metabolite

were subsequently applied to the design of ezitimibe, a marketed product of excellent potency.

2.4.4 Active Metabolites and PKPD Relationships

In addition to the potential discovery of improved compounds, the significant increase in effort directed towards the understanding of pharmacokinetic–pharmacodymanic (PKPD) relationships in drug discovery [110] provides a further rationale for active metabolite profiling. As animal models are typically used to assess PKPD relationships for novel compounds within the pharmaceutical industry, an adequate knowledge of the pharmacological activity of metabolites generated in the species in question is essential to ensure that the PKPD data is not open to misinterpretation. Such PKPD disconnects further confound the task of correlating PKPD relationships between animal species and humans thereby reducing confidence in predicted efficacy assessments made during drug discovery. A retrospective example of this principle is provided by the analgesic tramadol, the activity of which results from a combination of monoamine reuptake inhibition and μ-opioid receptor agonism, the latter largely attributable to the *O*-desmethyl metabolite (Fig. 28). Only following investigation of the activity of the major metabolic species was it possible to develop a robust PKPD model to describe the clinical response [111], showing the value of active metabolite assessment for predictive PKPD modeling.

2.5 Metabolite Safety

2.5.1 The Impact of Biotransformation on Safety Strategies

The conventional approach to underwrite the safety of novel compounds is well established, involving quantitation of parent compound exposure in toxicology and clinical studies in the hope of providing clear evidence of an exposure multiple in humans that engenders confidence in clinical safety. However, due to the many differences in clearance pathways between humans and toxicology species [112], additional considerations and strategies are required to provide confidence in the overall safety profile of compounds that undergo biotransformation in vivo. Although considerations around metabolite safety have been the topic of much discussion for many years, the publication of the Metabolites in Safety Testing

(MIST) guidelines by the FDA in 2008 [113] has initiated renewed focus on the key considerations and strategies to ensure that compounds entering drug development proceed with sufficient confidence in human safety.

2.5.2 Key Considerations to Understand the Safety Implications of Biotransformation Pathways

The key focus of the FDA MIST guidance document is on disproportionate circulating metabolites in humans at steady state, defined as metabolites whose plasma exposure following multiple dosing to humans is greater than the exposure in the circulation of toxicology species. Furthermore, the guidance introduces thinking around threshold levels of metabolite abundance, stating that circulating metabolites above 10% of the parent exposure require appropriate safety considerations while those below the 10% level do not, even if they are present at disproportionate levels in humans. Such numerical thinking, although intended to guide users around the appropriate response to minor circulating components, has in fact spawned a significant amount of inappropriate concern around the accuracy and precision of metabolite quantitation. A more holistic approach to MIST considerations is discussed in this section.

While the MIST document provides valuable context around metabolite considerations, it is intended to act as guidance around key principles rather than instruction as to strategic approach. As a result, in order to determine the most appropriate strategy for a given program, it is important that a thorough understanding of the relevance and importance of the metabolism data generated throughout discovery and development is developed and applied. Since the original PhRMA letter in 2002 [114], many diverse opinions have been expressed surrounding the MIST concept [115–118], giving rise to some additional considerations that can augment the principles of the FDA MIST document. One important point raised in the debate is that absolute, rather than relative abundance is a key reference point for metabolite safety, so that the total dose is appropriately factored in to the safety assessment. Simplistically, the point is that a metabolite accounting for 10% of the total dose in excreta following a 1 mg administration cannot be more significant in terms of human safety than if the same metabolite accounted for 1% of a 100 mg administration, as the body burden of metabolite is tenfold higher in the second scenario. Thus, to consider metabolites in terms of percentage alone can be misleading and absolute abundance is an important additional factor to consider. The same must be true for circulating metabolites, where the absolute concentration is a key parameter and should be considered in addition to the percentage of total radioactivity in plasma, the most typical assessment of metabolite quantity in definitive studies. The situation for circulating metabolites is further confounded by the fact that plasma concentrations are affected by the distribution of metabolites between plasma and other tissues, a property defined as the volume of distribution. The distribution volume is determined by the physicochemical properties of the metabolite in question and yields further

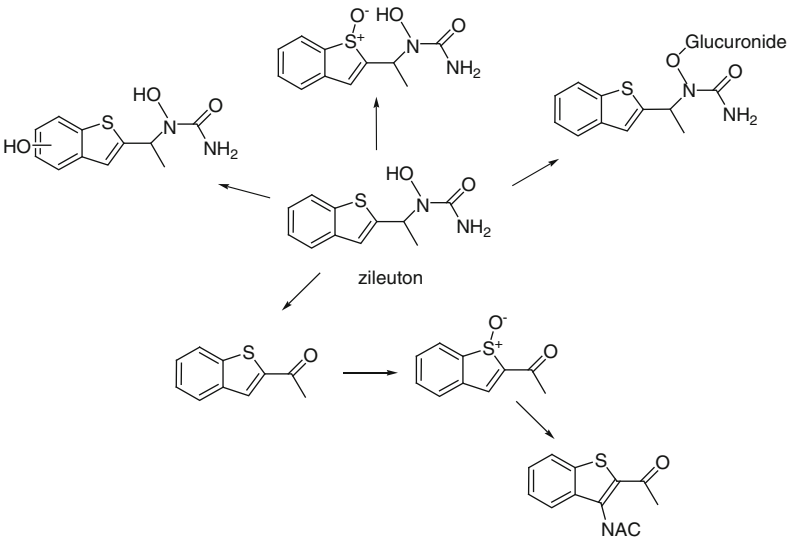


Fig. 29 Metabolic pathways of zileuton, including formation of an *N*-acetylthiophene metabolite via a reactive sulphoxide intermediate

uncertainty about the relationship between the concentration in human plasma and potential safety risk.

Another interesting consideration arising from the MIST discussion is that the specific structure of a metabolite is a key parameter in its safety assessment. For metabolites with a close structural resemblance to the parent compound (e.g., those resulting from demethylation, hydroxylation, and desaturation pathways), pharmacological activity both at the target receptor and at the nontarget receptors where the parent compound has been shown to have activity must be considered. Clearly, the presence and abundance of these metabolites in the circulation, as opposed to the excreta, is the key to safety assessments. It is unsurprising that metabolites which do not have a close structural resemblance to the parent compound are unlikely to have significant pharmacological activity at the target receptor. Therefore, while this does not mean that they can be ignored in terms of safety, different considerations are necessary. The most significant consideration is for metabolites that are indicative of the formation of reactive metabolic intermediates, the so-called smoking gun metabolites. While not of a direct safety concern, such components, including methylcatechols, mercapturic acids, and migrated acyl glucuronides, are nevertheless signals of potential safety risk arising from covalent binding of reactive metabolites to endogenous macromolecules. Smoking gun metabolites may not appear in the circulation at all but may well be present in excreta, as observed for the 5-lipooxygenase inhibitor zileuton, where an *N*-acetylthiophene metabolite was detected in urine, indicative of the formation of a reactive sulfoxide component (Fig. 29) [119]. The therapeutic use of zileuton is limited due to hepatotoxicity, possibly as a result of this metabolic pathway.

These considerations around metabolite abundance and structural relevance need to be a fundamental part of metabolite identification strategies throughout drug discovery and development to minimize the risk of compound attrition arising from inadequate human safety.

2.5.3 Strategies to Provide Confidence in Metabolite Safety

In order to minimize attrition resulting from safety considerations, it is appropriate to ensure that strategies are in place to adequately characterize metabolic pathways [120, 121]. At each stage of the metabolite identification process, the data may indicate that formal metabolite monitoring during clinical and safety programs is appropriate in order to give increased confidence in safety cover, in which case metabolite synthesis and quantitative analytical method development are required. Due to the resources needed to undertake formal metabolite monitoring, careful consideration should be given prior to initiating such an approach, including knowledge of the structure and pharmacological activity of the metabolite. In many cases, an initial semiquantitative approach in clinical studies may be appropriate to establish the approximate abundance of the metabolite in question and thereby determine the need for a formal monitoring approach.

A key focus of the metabolite characterization strategy is on the discovery phase, where the early identification of potential issues enables the design of more appropriate chemical matter. A suitable safety package in discovery would typically involve a cross-species comparison of in vitro data, using standard metabolizing systems, together with an assay to assess the propensity of compounds to undergo metabolism involving reactive intermediates (most commonly a glutathione adduction assay), which may lead to covalent binding in vivo. While the correlation between reactive metabolite formation and safety is not straightforward (Sect. 2.3), nevertheless, it remains prudent to attempt elucidation of any observed reactive mechanism pathways such that chemical redesign can be considered.

Definitive metabolism data is typically generated in humans and animals via the analysis of plasma and excreta following administration of radiolabeled material. As a result of the time and cost associated with the radiolabel synthesis and ADME package, however, such studies are often completed late in the drug development process, such that it is common for additional biotransformation studies to be conducted in early development in order to ensure that human safety is appropriately underwritten. Again, the key focus is on disproportionate human metabolites at steady state, such that the analysis of plasma samples from clinical and safety programs following multiple dosing is an appropriate strategy. The challenge is to provide sufficiently robust quantitative data to assess relative metabolite exposures in the absence of authentic standards or radiolabel, using alternative technologies such as UV [119], NMR spectroscopy [122, 123], fluorescence [124], chemiluminescent nitrogen detection (CLND) [125] or inductively coupled plasma mass spectrometry (ICP-MS) [126]. If sufficient thoroughness is applied to metabolism studies in early development, the definitive studies can reasonably be delayed until much later in the development continuum (often post a positive proof-of-concept

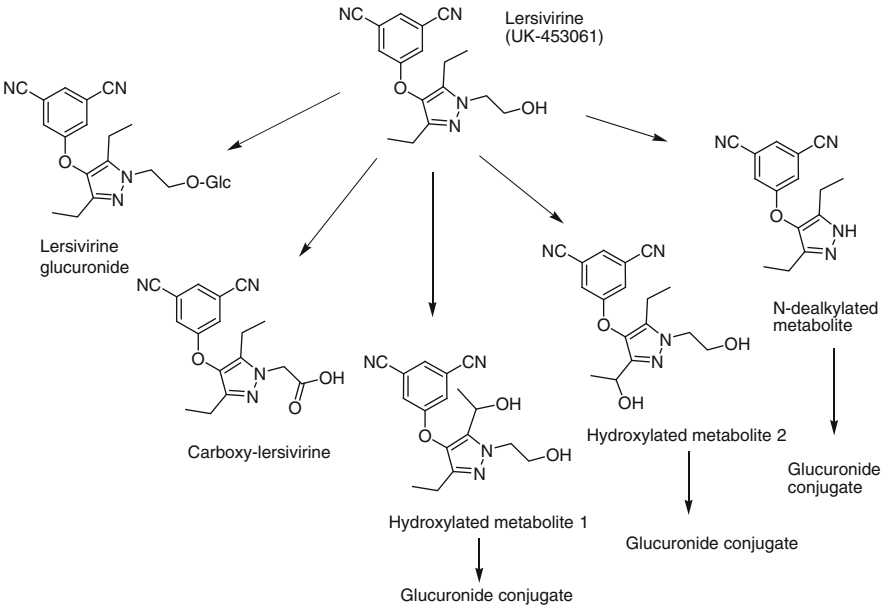


Fig. 30 Major metabolic pathways for lersivirine (UK-453,061)

read-out), when the use of radiolabeled material to enable the robust detection and quantitation of drug-related components and additional diligence on specific structural characterization are appropriate.

An example of a developing metabolite safety profile is illustrated by the non-nucleoside reverse transcriptase inhibitor (NNRTI), lersivirine (UK-453061) (Fig. 30). Early in vitro metabolism studies identified *N*-dealkylation and hydroxylation pathways in addition to glucuronidation of the primary alcohol functionality [127]. During early development, monitoring of the phase I oxidative products of metabolism was performed in an attempt to better understand the impact of the observed enzyme induction caused by the parent compound in toxicology studies [128]. Subsequent human metabolite scouting using phase I study samples showed a degree of similarity to the in vitro data, confirming the presence of at least two hydroxylated isomers as well as abundant glucuronic acid conjugates. In addition, the scouting data indicated the presence of a carboxylic acid metabolite and showed that the *N*-dealkylated metabolite was only present at trace levels in humans. These data resulted in additional work to confirm that the carboxylic acid metabolite was also present in toxicology species and to quantify the levels of the major glucuronide in order to aid understanding of the clearance pathways and to inform the drug–drug interaction strategy. A human radiolabeled ADME study with lersivirine confirmed the major routes of metabolism [129] and provided definitive structural and quantitative information on a range of minor components.

Although the focus of metabolism safety strategies is on the identification of disproportionate human metabolites following cross-species plasma analysis, the detection of such a metabolite does not necessarily lead to compound attrition. Simple

approaches to mitigate the risk include the use of an alternative animal species in the toxicology program where the metabolite in question is present at sufficient levels or administration of the metabolite to toxicology species in order to assess its safety. Further scholarship of the likely safety risk of the specific structure in question, including the structural relationship between parent compound and metabolite, and the disease area intended for the drug are additional considerations when assessing the significance of metabolite safety. Nevertheless, a sound understanding of the key considerations around safety and a holistic strategy to define metabolic pathways is advisable to optimize confidence in the safety of novel compounds.

3 Summary

It is clear from our own experience and from the general literature that the application of biotransformation studies and concepts has been readily embraced by the pharmaceutical industry as an approach to mitigating attrition. Biotransformation has evolved well beyond structural elucidation such that the real value of biotransformation data is in its application to other aspects of drug development. For example, identifying relevant clearance mechanisms directs scientists as to which in vitro models may be appropriate (or inappropriate) for making projections of human pharmacokinetics prior to first-in-human testing. Similarly, the value in identifying metabolic hot spots is not the structural characterization itself, but in enabling chemists' understanding for SAR development. Recently, much effort has gone into understanding reactive metabolites, and although the link between metabolism and safety is elusive, it remains prudent to continue to explore these relationships as early as possible in drug development, if for no other reason, than to give chemists the option of pursuing alternate chemical series devoid of such concerns if possible. As the industry seeks to reduce attrition, attention has been focused on more clear understanding of efficacy and biomarkers early in development and here once again, biotransformation finds a role to play. As PKPD models are developed, they can only be viewed as complete if they consider the entire disposition of a compound including its biotransformation and the potential contribution of pharmacologically active metabolites. And finally, of course, traditional safety is paramount to drug discovery and development and here again, the value in biotransformation studies lies in their ability to help underwrite and provide context for these studies. The understanding of metabolic profiles across species including humans is essential for the complete interpretation of regulatory toxicology studies.

Although biotransformation has generally been successfully incorporated earlier into the drug discovery paradigm, assembling the complete biotransformation package for a compound is still relatively labor- and time-intensive. However, as the discipline of biotransformation continues to evolve, e.g., new strategic approaches that may be implemented earlier, or new technologies such as

accelerator mass spectrometry that obviate the need for large quantities of radiolabeled material, it seems likely that biotransformation will make further inroads and contribute to understanding how to reduce drug attrition.

References

1. Riley RJ (2001) The potential pharmacological and toxicological impact of P450 screening. Curr Opin Drug Discov Devel 4:45–54
2. Strolin-Benedetti M, Whomsley R, Baltes E (2006) Involvement of enzymes other than CYPs in the oxidative metabolism of xenobiotics. Expert Opin Drug Metab Toxicol 2:895–921
3. Beedham C (1997) The role of non-P450 enzymes in drug oxidation. Pharm World Sci 19:255–263
4. Omiecinski CJ, Remel RP, Hosagrahara VP (1999) Concise review of the cytochrome P450s and their roles in toxicology. Toxicol Sci 48:151–156
5. Dalvie D, Obach RS, Kang P, Prakash CP, Loi C-M, Hurst S, Nedderman AN, Goulet L, Smith E, Bu H-Z, Smith DA (2009) Assessment of three human in vitro systems in the generation of major human excretory and circulating metabolites. Chem Res Toxicol 22:357–368
6. Lewis DFV (2003) Human cytochromes P450 associated with the phase 1 metabolism of drugs and other xenobiotics; A compilation of substrates and inhibitors of the CYP1, CYP2 and CYP3 families. Curr Med Chem 10:1955–1972
7. Trager WF (2006) Principles of drug metabolism 1: redox reactions. In: Testa B, van de Waterbeemd H (eds) Comprehensive medicinal chemistry, vol 5, 2nd edn. Elsevier, Oxford, pp 87–132
8. Davison AN (1958) Physiological role of monoamine oxidase. Physiol Rev 38:729–747
9. Castagnoli N, Trevor AJ, Singer TP, Sparatore A, Leung, L, Shinka T, Wu EY, Booth RG (1988) Metabolic studies on the nigrostriatal toxin MPTP. In: Progress in catecholamine research. Alan R. Liss, Inc., New York, pp 93–100
10. Binda C, Newton-Vinson P, Hubálek F, Edmondson DE, Mattevi A (2002) Structure of human monoamine oxidase B, a drug target for the treatment of neurological disorders. Nat Struct Biol 9:22–26
11. De Colibus L, Li M, Binda C, Lustig A, Edmondson DE, Mattevi A (2005) Three-dimensional structure of human monoamine oxidase A (MAO A): relation to the structures of rat MAO A and human MAO B. Proc Natl Acad Sci USA 102:12684–12689
12. Kalgutkar A, Dalvie D, Castagnoli N, Taylor TJ (2001) Interactions of nitrogen-containing xenobiotics with monoamine oxidase (MAO) isozymes A and B: SAR studies on MAO substrates and inhibitors. Chem Res Toxicol 1:1139–1162
13. Obach RS, Cox LM, Tremaine LM (2005) Sertraline is metabolized by multiple cytochrome P450 enzymes, monoamine oxidases, and glucuronyl transferases in human: an in vitro study. Drug Metab Dispos 33:262–270
14. Dixon CM, Park GR, Tarbit MH (1994) Characterisation of the enzyme responsible for the metabolism of sumatriptan in human liver. Biochem Pharmacol 47:1253–1257
15. Imamura Y, Wu X, Noda A, Noda H (2002) Side-chain metabolism of propranolol: involvement of monoamine oxidase and mitochondrial aldehyde dehydrogenase in the metabolism of N-desisopropylpropranolol to naphthoxylactic acid in rat liver. Life Sci 70:2687–2697
16. Garattini E, Fratelli M, Terao M (2008) Mammalian aldehyde oxidases: genetics, evolution and biochemistry. Cell Mol Life Sci 65:1019–1048
17. Moriyasu A, Sugihara K, Nakatani K, Ohta S, Kitamura S (2006) In vivo-in vitro relationship of methotrexate 7-hydroxylation by aldehyde oxidase in four different strain rats. Drug Metab Pharmacokinet 21:485–491

18. Chladek J, Martinkova J, Sispera L (1997) An in vitro study on methotrexate hydroxylation in rat and human liver. Physiol Res 46:371–379
19. Kitamura S, Sugihara K, Nakatani K, Ohta S, Oh-hara T, Ninomiya S, Green CE, Tyron CA (1999) Variation of hepatic methotrexate 7-hydroxylase activity in animals and humans. IUBMB Life 48:607–611
20. Beedham C, al-Tayib Y, Smith JA (1992) Role of guinea pig and rabbit hepatic aldehyde oxidase in oxidative in vitro metabolism of cinchona antimalarials. Drug Metab Dispos 20:889–895
21. Clarke SE, Harrell AW, Chenery RJ (1995) Role of aldehyde oxidase in the in vitro conversion of famciclovir to penciclovir in human liver. Drug Metab Dispos 23:251–254
22. Rashidi MR, Smith JA, Clarke SE, Beedham C (1997) In vitro oxidation of famciclovir and 6-deoxypenciclovir by aldehyde oxidase from human, guinea pig, rabbit, and rat liver. Drug Metab Dispos 25:805–813
23. Mitchell S (2008) Flavin mono-oxygenase (FMO) – the 'other' oxidase. Curr Drug Metab 9:280–284
24. Cashman JR (2008) Role of flavin-containing monooxgenase in drug development. Expert Opin Drug Metab Toxicol 12:1507–1521
25. Ziegler DM, Mitchell CH (1972) Microsomal oxidase. IV. Properties of a mixed-function amine oxidase isolated from pig liver microsomes. Arch Biochem Biophys 150:116–125
26. Cashman JR (2005) Some distinctions between flavin-containing and cytochrome P450 monooxygenases. Biochem Biophys Res Commun 338:599–604
27. Phillips IR, Francois AA, Shephard EA (2007) The flavin-containing monoooxygenases (FMOs): genetic variation and its consequences for the metabolism of therapeutic drugs. Curr Pharmacogenomics 5:292–313
28. Park SB, Jacob P III, Benowitz NL, Cashman JR (1993) Stereoselective metabolism of (S)-(−)-nicotine in humans: Formation of trans-(S)-(−)-nicotine N-1'-oxide. Chem Res Toxicol 6:880–888
29. Overby LH, Carver GC, Philpot RM (1997) Quantitation and kinetic properties of hepatic microsomal and recombinant flavin-containing monooxygenases 3 and 5 from humans. Chem Biol Interact 106:29–45
30. Kang J-H, Chung W-G, Lee K-H, Park C-S, Kang J-S, Shin I-C, Roh H-K, Dong M-S, Baek H-M, Cha Y-N (2000) Phenotypes of flavin-containing monooxygenase activity determined by ranitidine N-oxidation are positively correlated with genotypes of linked FMO3 gene mutations in a Korean population. Pharmacogenetics 10:67–78
31. Park C-S, Kang J-H, Chung W-G, Yi H-G, Pie J-E, Park D-K, Hines RN, McCarver DG, Cha Y-N (2002) Ethnic differences in allelic frequency of two flavin-containing monooxygenase 3 (FMO3) polymorphisms: linkage and effects on in vivo and in vitro FMO activities. Pharmacogenetics 12:77–80
32. Remmel R, Nagar S, Argikar U (2008) Conjugative metabolism of drugs. In: Zhang D, Zhu M, Humphreys WG (eds) Drug metabolism in drug design and development. Wiley, Hoboken, pp 37–88
33. Fisher MB, Paine MF, Strelevitz TJ, Wrighton SA (2001) The role of hepatic and extrahepatic UDP-glucuronosyltransferases in human drug metabolism. Drug Metab Rev 33:273–297
34. Sallustio BC (2008) Chapter 3: Glucuronidation-dependent toxicity and bioactivation. In: Fishbein JC (ed) Advances in molecular toxicology, vol 2. Elsevier, Amsterdam, pp 57–86
35. Fisher MB, VandenBranden M, Findlay K, Burchell B, Thummel KE, Hall SD, Wrighton SA (2000) Tissue distribution and interindividual variation in human UDP-glucuronosyltransferase activity: relationship between UGT1A1 promoter genotype and variability in a liver bank. Pharmacogenetics 10:727–739
36. Andersen G, Christrup L, Sjøgren P (2003) Relationships among morphine metabolism, pain and side effects during long-term treatment: an update. J Pain Symptom Manage 25:74–91

37. Itäaho K, Mackenzie PI, Ikushiro S, Miners JO, Moshe Finel M (2008) The configuration of the 17-hydroxy group variably influences the glucuronidation of β-estradiol and epiestradiol by human UDP-glucuronosyltransferases. Drug Metab Dispos 36:2307–2315
38. Smith DA, Schmid E, Jones B (2002) Do drug metabolism and pharmacokinetic departments make any contributions to drug discovery? Clin Pharmacokinet 41:1005–1019
39. Czodrowski P, Kriegl JM, Scheuerer S, Fox T (2009) Computational approaches to predict drug metabolism. Expert Opin Drug Metab Toxicol 5:15–27
40. van de Waterbeemd H, Gifford E (2003) ADMET in silico modelling: towards prediction paradise? Nat Rev Drug Discov 2:192–204
41. Zvinavashe E, Murk AJ, Rietjens IMCM (2008) Promises and pitfalls of quantitative structure-activity relationship approaches for predicting metabolism and toxicity. Chem Res Toxicol 21:2229–2236
42. McGuire JJ, Haile WH (2009) Metabolism-blocked antifolates as potential anti-rheumatoid arthritis agents: 4-amino-4-deoxy-5,8,10-trideazapteroyl-d, l-4'-methyleneglutamic acid (CH-1504) and its analogs. Biochem Pharmacol 77:1161–1172
43. Nair MG, Fayard ML, Lariccia JM, Amato AE, McGuire JJ, Galiva JH, Kisliuk RL (1999) Metabolism blocked classical folate analogue inhibitors of dihydrofolate reductase-1: synthesis and biological evaluation of mobiletrex. Med Chem Res 9:176–185
44. Cheng Y, Zhang F, Rano TA, Lu Z, Schleif WA, Gabryelski L, Olsen DB, Stahlhut M, Rutkowski CA, Lin JH, Jin L, Emini EA, Chapman KT, Tata JR (2002) Indinavir analogues with blocked metabolism sites as HIV protease inhibitors with improved pharmacological profiles and high potency against PI-Resistant viral strains. Bioorg Med Chem Lett 12:2419–2422
45. Middleton DS, Andrews M, Glossop P, Gymer G, Hepworth D, Jessiman A, Johnson PS, MacKenny M, Stobie A, Tang K, Morgan P, Jones B (2008) Designing rapid onset selective serotonin re-uptake inhibitors. Part 3: site-directed metabolism as a strategy to avoid active circulating metabolites: Structure–activity relationships of (thioalkyl)phenoxy benzylamines. Bioorg Med Chem Lett 18:5303–5306
46. Moorjani M, Luo Z, Lin E, Vong BG, Chen Y, Zhang X, Rueter JK, Gross RS, Lanier MC, Tellew JE, Williams JP, Lechner SM, Malany S, Santos M, Crespo MI, Díaz J-L, Saunders J, Slee DH (2008) 2,6-Diaryl-4-acylaminopyrimidines as potent and selective adenosine A2A antagonists with improved solubility and metabolic stability. Bioorg Med Chem Lett 18:5402–5405
47. Park BK, Kitteringham NR, O'Neill PM (2001) Metabolism of fluorine-containing drugs. Annu Rev Pharmacol Toxicol 41:443–470
48. Müller K, Faeh C, Diederich F (2007) Fluorine in pharmaceuticals: looking beyond intuition. Science 317:1881–1886
49. Shafer CM, Lindvall M, Bellamacina C, Gesner TG, Yabannavar A, Jia W, Lin S, Walter A (2008) 4-(1H-Indazol-5-yl)-6-phenylpyrimidin-2(1H)-one analogs as potent CDC7 inhibitors. Bioorg Med Chem Lett 18:4482–4485
50. Kramer JA, Sagartz JE, Morris DL (2007) The application of discovery toxicology and pathology towards the design of safer pharmaceutical lead candidates. Nat Rev Drug Discov 6:636–649
51. Olson H, Betton G, Robinson D et al (2000) Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharmacol 32:56–67
52. Miller EC, Miller JA (1966) Mechanisms of chemical carcinogenesis: nature of proximate carcinogens and interactions with macromolecules. Pharmacol Rev 18:805–838
53. Mitchell JR, Jollow DJ, Potter WZ, Davis DC, Brodie BB (1973) Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. J Pharmacol Exp Ther 187:185–194
54. Jollow DJ, Mitchell JR, Potter WZ, Davis DC, Gillette JR, Brodie BB (1973) Acetaminophen-induced hepatic necrosis. II. Role of covalent binding in vivo. J Pharmacol Exp Ther 187:195–202

55. Potter WZ, Davis DC, Mitchell JR, Jollow DJ, Gillette JR, Brodie BB (1973) Acetaminophen-induced hepatic necrosis. III. Cytochrome P-450-mediated covalent binding in vitro. J Pharmacol Exp Ther 187:203–210
56. Mitchell JR, Jollow D, Potter WZ, Gillette JR, Brodie BB (1973) Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. J Pharmacol Exp Ther 187:211–217
57. Dahlin DC, Miwa GT, Lu AY, Nelson SD (1984) N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. Proc Natl Acad Sci USA 81:1327–1331
58. Zhao Z, Baldo BA, Rimmer J (2002) Beta-Lactam allergenic determinants: fine structural recognition of a cross-reacting determinant on benzylpenicillin and cephalothin. Clin Exp Allergy 32:1644–1650
59. Hess DA, Sisson ME, Suria H, Wijsman J, Puvanesasingham R, Madrenas J, Rieder MJ (1999) Cytotoxicity of sulfonamide reactive metabolites: apoptosis and selective toxicity of CD8(+) cells by the hydroxylamine of sulfamethoxazole. FASEB J 13:1688–1698
60. Lecoeur S, Andre C, Beaune PH (1996) Tienilic acid-induced autoimmune hepatitis: anti-liver and-kidney microsomal type 2 autoantibodies recognize a three-site conformational epitope on cytochrome P4502C9. Mol Pharmacol 50:326–333
61. Bourdi M, Gautier JC, Mircheva J, Larrey D, Guillouzo C, Andre C, Belloc C, Beaune PH (1992) Anti-liver microsomes autoantibodies and dihydralazine-induced hepatitis: specificity of autoantibodies and inductive capacity of the drug. Mol Pharmacol 42:280–285
62. Bourdi M, Chen W, Peter RM, Martin JL, Buters JT, Nelson SD, Pohl LR (1996) Human cytochrome P450 2E1 is a major autoantigen associated with halothane hepatitis. Chem Res Toxicol 9:1159–1166
63. Kalgutkar AS, Soglia JR (2005) Minimising the potential for metabolic activation in drug discovery. Expert Opin Drug Metab Toxicol 1:91–142
64. Kalgutkar AS, Gardner I, Obach RS et al (2005) A comprehensive listing of bioactivation pathways of organic functional groups. Curr Drug Metab 6:161–225
65. Correia MA, Krowech G, Caldera-Munoz P, Yee SL, Straub K, Castagnoli N Jr (1984) Morphine metabolism revisited. II. Isolation and chemical characterization of a glutathionylmorphine adduct from rat liver microsomal preparations. Chem Biol Interact 51:13–24
66. Soglia JR, Harriman SP, Zhao S, Barberia J, Cole MJ, Boyd JG, Contillo LG (2004) The development of a higher throughput reactive intermediate screening assay incorporating micro-bore liquid chromatography-micro-electrospray ionization-tandem mass spectrometry and glutathione ethyl ester as an in vitro conjugating agent. J Pharm Biomed Anal 36:105–116
67. Baillie TA, Davis MR (1993) Mass spectrometry in the analysis of glutathione conjugates. Biol Mass Spectrom 22:319–325
68. Sahali-Sahly Y, Balani SK, Lin JH, Baillie TA (1996) In vitro studies on the metabolic activation of the furanopyridine L-754,394, a highly potent and selective mechanism-based inhibitor of cytochrome P450 3A4. Chem Res Toxicol 9:1007–1012
69. Olsen R, Molander P, Øvrebø S et al (2005) Reaction of glyoxal with 2'-deoxyguanosine, 2'-deoxyadenosine, 2'-deoxycytidine, cytidine, thymidine, and calf thymus DNA: identification of DNA adducts. Chem Res Toxicol 18:730–739
70. Gorrod JW, Whittlesea CM, Lam SP (1991) Trapping of reactive intermediates by incorporation of 14C-sodium cyanide during microsomal oxidation. Adv Exp Med Biol 283:657–664
71. Argoti D, Liang L, Conteh A, Chen L, Bershas D, Yu CP, Vouros P, Yang E (2005) Cyanide trapping of iminium ion reactive intermediates followed by detection and structure identification using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Chem Res Toxicol 18:1537–1544
72. Walker GS, Atherton J, Bauman J, Kohl C, Lam W, Reily M, Lou Z, Mutlib A (2007) Determination of degradation pathways and kinetics of acyl glucuronides by NMR spectroscopy. Chem Res Toxicol 20:876–886

73. Johnson CH, Wilson ID, Harding JR, Stachulski AV, Iddon L, Nicholson JK, Lindon JC (2007) NMR spectroscopic studies on the in vitro acyl glucuronide migration kinetics of Ibuprofen ((+/−)-(R, S)-2-(4-isobutylphenyl) propanoic acid), its metabolites, and analogues. Anal Chem 79:8720–8727
74. Evans DC, Watt AP, Nicoll-Griffith DA, Baillie TA (2004) Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. Chem Res Toxicol 17:3–16
75. Kalgutkar AS, Dalvie DK, Aubrecht J et al (2007) Genotoxicity of 2-(3-chlorobenzyloxy)-6-(piperazinyl)pyrazine, a novel 5-hydroxytryptamine2c receptor agonist for the treatment of obesity: role of metabolic activation. Drug Metab Dispos 35:848–858
76. Amos HE, Lake BG, Artis J (1978) Possible role of antibody specific for a practolol metabolite in the pathogenesis of oculomucocutaneous syndrome. Br Med J 1:402–404
77. Orton C, Lowery C (1981) Practolol metabolism. III. Irreversible binding of [14C]practolol metabolite(s) to mammalian liver microsomes. J Pharmacol Exp Ther 219:207–212
78. Borchard U (1990) Pharmacokinetics of beta-adrenoceptor blocking agents: clinical significance of hepatic and/or renal clearance. Clin Physiol Biochem 8(suppl 2):28–34
79. Satoh H, Martin BM, Schulick AH, Christ DD, Kenna JG, Pohl LR (1989) Human anti-endoplasmic reticulum antibodies in sera of patients with halothane-induced hepatitis are directed against a trifluoroacetylated carboxylesterase. Proc Natl Acad Sci USA 86:322–326
80. Njoku D, Laster MJ, Gong DH, Eger EI II, Reed GF, Martin JL (1997) Biotransformation of Halothane, enflurane, isoflurane, and desflurane to trifluoroacetylated liver proteins: association between protein acylation and hepatic injury. Anesth Analg 84:173–178
81. Jacobsen W, Kuhn B, Soldner A, Kirchner G, Sewing KF, Kollman PA, Benet LZ, Christians U (2000) Lactonization is the critical first step in the disposition of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor atorvastatin. Drug Metab Dispos 28:1369–1378
82. Prueksaritanont T, Subramanian R, Fang X, Ma B, Qiu Y, Lin JH, Pearson PG, Baillie TA (2002) Glucuronidation of statins in animals and humans: a novel mechanism of statin lactonization. Drug Metab Dispos 30:505–512
83. Savi P, Pereillo JM, Uzabiaga MF, Combalbert J, Picard C, Maffrand JP, Pascal M, Herbert JM (2000) Identification and biological activity of the active metabolite of clopidogrel. Thromb Haemost 84:891–896
84. Savi P, Zachayus JL, Delesque-Touchard N et al (2006) The active metabolite of Clopidogrel disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts. Proc Natl Acad Sci USA 103:11069–11074
85. Herbert JM, Savi P (2003) P2Y12, a new platelet ADP receptor, target of clopidogrel. Semin Vasc Med 3:113–122
86. Olbe L, Carlsson E, Lindberg P (2003) A proton-pump inhibitor expedition: the case histories of omeprazole and esomeprazole. Nat Rev Drug Discov 2:132–139
87. Fellenius E, Berglindh T, Sachs G, Olbe L, Elander B, Sjöstrand SE, Wallmark B (1981) Substituted benzimidazoles inhibit gastric acid secretion by blocking (H^+ + K^+)ATPase. Nature 290:159–161
88. Durand A, Thenot JP, Bianchetti G, Morselli PL (1992) Comparative pharmacokinetic profile of two imidazopyridine drugs: zolpidem and alpidem. Drug Metab Rev 24:239–266
89. Chen Q, Ngui JS, Doss GA et al (2002) Cytochrome P450 3A4-mediated bioactivation of raloxifene: irreversible enzyme inhibition and thiol adduct formation. Chem Res Toxicol 15:907–914
90. Kemp DC, Fan PW, Stevens JC (2002) Characterization of raloxifene glucuronidation in vitro: contribution of intestinal metabolism to presystemic clearance. Drug Metab Dispos 30:694–700
91. Obach RS, Kalgutkar AS, Soglia JR, Zhao SX (2008) Can in vitro metabolism-dependent covalent binding data in liver microsomes distinguish hepatotoxic from nonhepatotoxic drugs? An analysis of 18 drugs with consideration of intrinsic clearance and daily dose. Chem Res Toxicol 21:1814–1822

92. Bauman JN, Kelly JM, Tripathy S, Zhao SX, Lam WW, Kalgutkar AS, Obach RS (2009) Can in vitro metabolism-dependent covalent binding data distinguish hepatotoxic from nonhepatotoxic drugs? An analysis using human hepatocytes and liver S-9 fraction. Chem Res Toxicol 22:332–340
93. Ring BJ, Patterson BE, Mitchell MI et al (2005) Effect of tadalafil on cytochrome P450 3A4-mediated clearance: studies in vitro and in vivo. Clin Pharmacol Ther 77:63–75
94. Erve JC, Vashishtha SC, DeMaio W, Talaat RE (2007) Metabolism of prazosin in rat, dog, and human liver microsomes and cryopreserved rat and human hepatocytes and characterization of metabolites by liquid chromatography/tandem mass spectrometry. Drug Metab Dispos 35:908–916
95. Nakayama S, Atsumi R, Takakusa H, Kobayashi Y, Kurihara A, Nagai Y, Nakai D, Okazaki O (2009) A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding. Drug Metab Dispos 37:1970–1977
96. Hughes AR, Brothers CH, Mosteller M, Spreen WR, Burns DK (2009) Genetic association studies to detect adverse drug reactions: abacavir hypersensitivity as an example. Pharmacogenomics 10:225–233
97. Brynne N, Stahl MMS, Hallen B, Edlund PO, Palmer L, Hoglund P, Gabrielsson J (1997) Pharmacokinetics and pharmacodynamics of tolterodine in man. A new drug for the treatment of urinary bladder overactivity. Int J Clin Pharmacol Ther 35:287–295
98. Nilvebrant L, Gillberg P-G, Sparf B (1997) Antimuscarinic potency and bladder selectivity of PNU-200577, a major metabolite of tolterodine. Pharmacol Toxicol (Copenhagen) 81:169–172
99. Connolly HM, Crary JL, McGoon MD, Hensrud DD, Edwards BS, Edwards WD, Schaff HV (1997) Valvular heart disease associated with fenfluramine-phentermine. N Engl J Med 337:581–588
100. Weissman NJ (2001) Appetite suppressants and valvular heart disease. Am J Med Sci 321:285–291
101. Roth BL (2007) Drugs and valvular heart disease. N Engl J Med 356:6–9
102. De Boer AR, Letzel T, Van Elswijk DA, Lingeman H, Niessen WMA, Irth H (2004) On-line coupling of high-performance liquid chromatography to a continuous-flow enzyme assay based on electrospray ionization mass spectrometry. Anal Chem 76:3155–3161
103. De Boer AR, Alcaide-Hidalgo JM, Krabbe JG, Kolkman J, Van Emde Boas CN, Niessen WMA, Lingeman H, Irth H (2005) High-temperature liquid chromatography coupled on-line to a continuous-flow biochemical screening assay with electrospray ionization mass spectrometric detection. Anal Chem 77:7894–7900
104. Van Liempd SM, Kool J, Niessen WMA, van Elswijk DE, Irth H, Vermeulen NPE (2006) On-line formation, separation, and estrogen receptor affinity screening of cytochrome P450-derived metabolites of selective estrogen receptor modulators. Drug Metab Dispos 34:1640–1649
105. Van Liempd SM, Kool J, Meerman JH, Irth H, Vermeulen NP (2007) Metabolic profiling of endocrine-disrupting compounds by on-line cytochrome P450 bioreaction coupled to on-line receptor affinity screening. Chem Res Toxicol 20:1825–1832
106. Yun CH, Okerholm RA, Guengerich FP (1993) Oxidation of the antihistaminic drug terfenadine in human liver microsomes. Role of cytochrome P-450 3A(4) in N-dealkylation and C-hydroxylation. Drug Metab Dispos 21:403–409
107. Honig PK, Wortham DC, Zamani K, Conner DP, Mullin JC, Cantilena LR (1993) Terfenadine-ketoconazole interaction. Pharmacokinetic and electrocardiographic consequences. J Am Med Assoc 269:1513–1518
108. Van Heek M, France CF, Compton DS, McLeod RL, Yumibe NP, Alton KB, Sybertz EJ, Davis HR Jr (1997) In vivo metabolism-based discovery of a potent cholesterol absorption inhibitor, SCH 58235, in the rat and rhesus monkey through identification of the active metabolites of SCH 48461. J Pharmacol Exp Ther 283:157–163
109. Clader JW (2004) The discovery of ezetimibe: a view from outside the receptor. J Med Chem 47:1–9

110. Danhof M, Van der Graaf PH, Jonker DM, Visser SAG, Zuideveld KP (2006) Mechanism-based pharmacokinetic-pharmacodynamic modeling for the prediction of in vivo drug concentration-effect relationships- application in drug candidate selection and lead optimization. Compr Med Chem II 5:885–908
111. Beier H, Garrido MJ, Christoph T, Kasel D, Troconiz IF (2008) Semi-mechanistic pharmacokinetic/pharmacodynamic modelling of the antinociceptive response in the presence of competitive antagonism: the interaction between tramadol and its active metabolite on m-opioid agonism and monoamine reuptake inhibition, in the rat. Pharm Res 25:1789–1797
112. Smith DA (1991) Species differences in metabolism and pharmacokinetics: are we close to an understanding? Drug Metab Rev 23:355–373
113. FDA Guidance for Industry. Safety Testing of Drug Metabolites (2008) http://www.fda.gov/cder/guidance/6897fnl.pdf
114. Baillie TA, Cayen MN, Fouda H, Gersons RJ, Green JD, Grossman SJ, Klunk LJ, LeBlanc B, Perkins DG, Shipley LA (2002) Drug metabolites in safety testing. Toxicol Appl Pharmacol 182:188–196
115. Smith DA, Obach RS (2005) Seeing through the mist: abundance versus percentage. Commentary on metabolites in safety testing. Drug Metab Dispos 33:1409–1417
116. Smith DA, Obach RS (2006) Metabolites and Safety: what are the concerns and how should we address them? Chem Res Toxicol 19:1570–1579
117. Humphreys WG, Unger SE (2006) Safety assessment of drug metabolites: characterization of chemically stable metabolites. Chem Res Toxicol 19:1564–1569
118. Baillie TA (2009) Approaches to the assessment of stable and chemically reactive drug metabolites in early clinical trials. Chem Res Toxicol 22:263–266
119. Joshi EM, Heasley BH, Chordia MD, Macdonald TL (2004) In vitro metabolism of 2-acetylbenzothiophene: relevance to zileuton hepatotoxicity. Chem Res Toxicol 17:137–143
120. Walker DK, Brady J, Dalvie D, Davis J, Dowty M, Duncan JN, Nedderman A, Obach RS, Wright P (2009) A holistic strategy for characterising the safety of metabolites through drug discovery and development. Chem Res Toxicol 22:1653–1662
121. Viswanathan K, Babalola K, Wang J, Espina R, Yu L, Adedoyin A, Talaat R, Mutlib A, Scatina J (2009) Obtaining exposures of metabolites in preclinical species through plasma pooling and quantitative NMR: addressing metabolites in safety testing (MIST) guidance without using radiolabeled compounds and chemically synthesized metabolite standards. Chem Res Toxicol 22:311–322
122. Dear GJ, Roberts AD, Beaumont C, North SE (2008) Evaluation of preparative high performance liquid chromatography and cryoprobe-nuclear magnetic resonance spectroscopy for the early quantitative estimation of drug metabolites in human plasma. J Chromatogr B: Anal Technol Biomed Life Sci 876:182–190
123. Espina R, Yu L, Wang J, Tong Z, Vashishtha S, Talaat R, Scatina J, Mutlib A (2009) Nuclear magnetic resonance spectroscopy as a quantitative tool to determine the concentrations of biologically produced metabolites: implications in metabolites in safety testing. Chem Res Toxicol 22:299–310
124. Fang J, Semple HA, Song J (2004) Determination of metoprolol and its four metabolites in dog plasma. J Chromatogr B: Anal Technol Biomed Life Sci 809:9–14
125. Taylor EW, Jia W, Bush M, Dollinger GD (2002) Accelerating the drug optimisation process: identification, structure elucidation and quantification of in vivo metabolites using stable isotopes with LC/MS^n and the chemiluminescent nitrogen detector. Anal Chem 74:3232–3238
126. Axelsson B-O, Jornten-Karlsson M, Michelsen P, Abou-Shakra F (2001) The potential of inductively coupled plasma mass spectrometry detection for high-performance liquid chromatography combined with accurate mass measurement or organic pharmaceutical compounds. Rapid Commun Mass Spectrom 15:375–385
127. Allan G, Davis J, Dickins M, Gardner I, Jenkins T, Jones H, Webster R, Westgate H (2008) Pre-clinical pharmacokinetics of UK-453,061, a novel non-nucleoside reverse transcriptase inhibitor (NNRTI), and use of in silico physiologically based prediction tools to predict the oral pharmacokinetics of UK-453,061 in man. Xenobiotica 38:620–640

128. Walker DK, Davis J, Houle C, Gardner IB, Webster R (2009) Species differences in the multiple dose pharmacokinetics of the non-nucleoside reverse transcriptase inhibitor (NNRTI) UK-453,061 in animals and man – implications for safety considerations. Xenobiotica 39:534–543
129. Vourvahis M, Gleave M, Nedderman AN, Hyland R, Gardner I, Howard M, Kempshall S, Collins C, LaBadie R (2010) Excretion and metabolism of lersivirine (5-{[3,5-diethyl-1-(2-hydroxyethyl)(3,5–14C2)-1H-pyrazol-4-yl]oxy}benzene-1,3-dicarbonitrile), a next-generation non-nucleoside reverse transcriptase inhibitor, after administration of [^{14}C] Lersivirine to healthy volunteers. Drug Metab Dispos 38:789–800

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Reducing Drug Attrition: Safety Pharmacology

Peter Siegl

Abstract Safety pharmacology studies have been used to support drug discovery and development programs by many sponsors for decades. The objectives of these studies are to identify and characterize pharmacology activities of drug candidates that can contribute to adverse effects and safety of clinical trial participants and patients. International guidelines for safety pharmacology were adopted in 2001 and 2005, and results from these studies are now a component of almost all regulatory submissions. By insuring the quality of the data from these studies and effectively communication results in terms of a risk assessment, the safety pharmacology package can be used to identify drug candidates with lower risk for attrition and to mitigate or better manage safety risks during clinical development.

Keywords Drug safety, ICH guidelines S7A and S7B, Preclinical drug development, Safety pharmacology

Contents

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1 Introduction

Drug discovery and development is a high-risk enterprise where greater than 95% of drug candidates that enter development fail to achieve market approval [1–3]. While this is widely recognized and root causes for attrition are the subject of many publications and meetings, the probability of success (POS) for new drug candidates has not significantly improved over the past 10 years. At the same time, the public, government, and healthcare payers are challenging the pharmaceutical industry to deliver new drugs at reduced prices and with higher safety standards. Combined with elevated costs of research and high attrition rates for both drug candidates and market drugs, this presents a momentous challenge for the pharmaceutical industry.

Many of the larger pharmaceutical (Pharma) organizations have responded to the lower productivity of drug development by increasing the number of drug candidates they progress into development ("more shots on goal"). This is combined with an objective to detect liabilities and abandon candidates earlier in development. In this scenario, the goal is to increase attrition rates for candidates with liabilities as early as possible in order to progress more candidates into the pipeline. The premise is that drug development failures are stochastic events and it is impractical to predict clinical efficacy and anticipate safety liabilities with confidence. A quote from the past head of research at Pfizer reflects this thinking; "Limiting your shots by assuming that you can predict winners may ultimately prove to be a flawed strategy" (http://johnlamattina.wordpress.com/2011/05/26/why-taking-shots-on-goal-matters-to-rd/ 2011). This approach has not been successful, and most now appreciate that it is not solely the number of shots on goal but also the quality of the shots. The obvious solution is to advance higher quality drug candidates into development and more effectively manage their development.

Accurately predicting the attrition risk or inversely, the POS for drug candidates to achieve market approval enables sponsors to more effectively manage their pipelines and resources. This will, in turn, facilitate a greater number of safer and more effective drugs reaching patients. This vision is reflected in both the "Challenge and Opportunity on the Critical Path to New Medical Products" by the FDA (http://www.fda.gov/ScienceResearch/SpecialTopics/CriticalPathInitiative/CriticalPathOpportunitiesReports/ucm077262.htm) [4] and

"The European Medicines Agency Road Map to 2010: Preparing the Ground for the Future" (http://www.emea.europa.eu/docs/en_GB/document_library/Report/2009/10/WC500004903.pdf). The premise for these initiatives is that sponsors need to leverage advances in technology, such as predictive toxicology and biomarkers, and doing so will lead to greater knowledge and better monitoring of safety and efficacy of drug candidates. Results from safety pharmacology studies are important contributors to assessing safety and risk for attrition earlier in drug development and improving quality of drug development candidates. The success of these strategies will be measurable by a reduction in attrition rates.

To more accurately predict the POS for drug development projects, we need to maximize the quality of the information and data from safety pharmacology studies as well as acknowledge there will be gaps in our knowledge of relevant pharmacology and pathophysiology. In other words, a realistic comprehension of the scientific strength and limitations of available data/information is essential for estimating POS and provides an important opportunity to reduce drug attrition in clinical development and beyond.

2 Definitions of Safety Pharmacology

The objectives of safety pharmacology studies are to identify and characterize pharmacological activities of drug candidates which could impact the safety and tolerability. These include both targeted and off-target activities. The term was first introduced by Professor Gerhard Zbinden [5] who recognized that there are significant adverse drug reactions that cannot be predicted from results of conventional toxicology studies. Toxicology studies focus primarily on endpoints such as mortality, physical signs, and histomorphology. Safety pharmacology studies interrogate indices of physiological function and the pharmacodynamic consequences of acute or chronic drug administration. Single-dose protocols are most often used, and responses measured in safety pharmacology studies are not usually accompanied by histomorphological changes. Employment of physiological endpoints to assess the potential direct effects of a test substance on organ function (e.g., cardiovascular, respiratory, central nervous system, and renal) compliments the traditional toxicology package by providing more complete information for the human safety risk assessment. In the absence of safety pharmacology data, many of these potential adverse activities would not be detected until the drug candidate is in clinical studies.

Safety pharmacology also differs from traditional toxicology in that the treatment-related activities identified in safety pharmacology studies may not by themselves be adverse events. It some cases, it is when the observed activity is combined with other risk factors or occurs in a subset of susceptible patients, that the activity becomes a clinical safety concern. For example, for drugs that inhibit the cardiac potassium current, I_{Kr} (also referred to as the hERG channel), the resulting delay in ventricular repolarization (reflected as a prolongation of the QT

interval on the surface electrocardiogram) is not an adverse event and may occur in a majority of patients without any adverse consequence. However, delayed ventricular repolarization is a risk factor that, when combined with other risk factors, can cause cardiac arrhythmias in a very small subset of patients [6]. Therefore, it is appropriate to evaluate findings from safety pharmacology studies in terms of their relative risk in the context of proposed clinical use and not merely on the acute, observed effects.

Safety pharmacology data can be a valuable contributor to understanding the benefit to risk proposition for drug candidates such that the most effective and safest drugs can be made available to patients as well as to help insure that the drug is used in a manner that maximizes its safety and benefit to patients. The safety pharmacology package should not be limited to the core assay described in the regulatory guidelines (http://www.ich.org/products/guidelines/safety/article/safety-guidelines.html). It is important to design safety pharmacology studies and interpret the results in the context of these broader objectives of the development program and with information from other sources (e.g., drug discovery, toxicology, clinical pharmacology) to better understand the overall pharmacology of the drug candidate. Together all this information is used to develop an integrated risk assessment (see Sect. 5.2) which enables better decisions for drug development with respect to advancing the best candidates and minimizing the risk for attrition at later stages of development.

In addition to identifying potential liabilities, results from safety pharmacology studies can be used to define the safety of a drug candidate. It can be more challenging to demonstrate that a drug is safe (without liabilities, lower attrition risk) even though this is the goal of successful drug development programs. Evidence for absence of activity is accomplished with sensitive assays and comparing results with the drug candidate to those with appropriate reference drugs (see [7]). In designing a safety pharmacology strategy, it is important to use well-characterized assays and provide data with reference agents as well as the test substance to clearly document the safety as well as potential liabilities of the drug development candidate.

3 Safety Pharmacology Regulatory Guidelines

The term safety pharmacology first appeared in international guidelines in the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) topics S6 and M3 (http://www.ich.org/products/guidelines/safety/article/safety-guidelines.html). In the M3 guideline, three types of pharmacological activities are described. *Primary pharmacology* refers to the pharmacodynamic activities that confer therapeutic benefit to the patient. Pharmacodynamic activities without therapeutic benefit are divided into two categories, *secondary pharmacology* and *safety pharmacology*. The difference between the two is that secondary pharmacological activity does not have an impact

on safety, while safety pharmacological activity does. Secondary and safety pharmacological activities can be either mechanism based (i.e., same mechanism as that targeted for therapeutic benefit) or off target. By having the two categories of secondary and safety pharmacology, it suggests that regulators might accept drug candidates with pharmacodynamic activities that are neutral on safety and efficacy. However, it is difficult to know with absolute certainty which unanticipated pharmacological activities are without safety implications in broad patient populations. Since the consequence of an unanticipated activity on human safety might not be apparent until late in clinical development, or even in post-marketing surveillance, even secondary pharmacological activities may carry some attrition risk for drug development. Ideally, lead optimization in drug discovery programs will maximize selectivity of drug candidates against the majority of off-target activities. Therefore, distinguishing between secondary pharmacology and safety pharmacology may have minimal importance in the overall risk evaluation, and for the purposes of discussion in this chapter, all pharmacodynamic activities not contributing to therapeutic benefit will be considered in safety pharmacology assessments.

Prior to their reference in ICH guidelines, safety pharmacology-type studies were referred to by several names. In the Japanese guidelines, these type studies are called General Pharmacology Studies (Japanese Guidelines for Non-clinical Studies of Drugs Manual 1995. Yakuji Nippo, Tokyo). Safety pharmacology has also been referred to as Ancillary Pharmacology by some organizations. The concept is that these studies are looking for and characterize activities that are "ancillary" to the primary pharmacological activity. Therefore, ancillary and general pharmacological studies have been used to by some to define the drug candidate's selectivity profile and contribute to overall risk assessment of drug candidates prior to implementation of regulatory guidelines.

In 2001, specific guidelines for safety pharmacology studies were implemented in the United States, European Union, and Japan (ICH topic S7A, "Safety Pharmacology Studies for Human Pharmaceuticals"). A subsequent ICH guideline focusing on risk assessment for delayed ventricular repolarization (QT interval prolongation) and the associated cardiac arrhythmia, Torsade de Pointes, was implemented in 2005 (ICH topic S7B, "The Non-Clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals") as well as a related clinical guidance (ICH topic E14, "Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential for Non-Arrhythmic Drugs").

The international safety pharmacology guidelines ICH S7A and ICH S7B recommend core assays and testing conditions that, when followed, support a safety package that will meet the regulatory requirements of most regions. It is important to point out that these are "guidelines" and not "rules"; however, as with most regulatory guidelines, by not providing data from recommended core assays, the regulator may have difficulty comparing the results with those from other compounds. If one employs safety pharmacology assays that differ from those recommended, it is particularly important to justify the choice of assay(s) as well as provide data from control and reference compounds in the same assay to support

your safety risk assessment and aid the reviewer in his/her evaluation. An important recommendation in the guideline that is often missed is to define the sensitivity and specificity of the assays using with positive and negative control agents. This is particularly important in supporting a conclusion that the drug candidate has no activity in the assay. The guidelines also recommend use of follow-up assays to further characterize and support conclusions; however, sponsors rarely include results from follow-up assays in safety pharmacology packages. Finally, the guidelines recommend that the sponsor provides an integrated risk assessment to support conclusions related to patient safety. Burden of proof for the safety of a drug candidate is the responsibility of the sponsor so one should strive to build a solid, defendable scientific case in order to support claims using tools recommended in the guidelines.

Some sponsors limit their investigation to the core safety pharmacology assays and report results solely to comply with the regulatory guidelines. This approach minimizes use of resources but can miss important opportunities to leverage the data that will impact attrition. The information from safety pharmacology studies can be used to further define and support the selectivity of a drug candidate for its primary activity. Results should also be referenced in reports of toxicology studies to build a stronger case for safety. Safety pharmacology data are often valuable in the design and interpretation of early clinical studies. Follow-up safety pharmacology studies can be carried out when clinical data are available to further understand or support conclusions from the clinical investigation. Results from a well-designed safety pharmacology package should be integrated into various sections of regulatory packages to strengthen the scientific level of safety assessment.

4 Basic Concepts and General Approaches to Safety Pharmacology Studies

As with all aspects of drug discovery and development, safety pharmacology is based on the principles that a threshold concentration must be achieved at the site of action for a drug to elicit a biological response and the magnitude of the response is a function of the drug concentrated at the site of action. The threshold concentration below which a drug can elicit a response is called the No Effect Level (NOEL). The magnitude of a drug response is usually described as a percentage of its maximum response. These concepts apply to both beneficial and adverse effects of drugs.

Results from well designed and characterized in vitro assays allow one to determine the threshold concentration and concentration–response relationship of a drug candidate at its site of action. High-throughput, relatively inexpensive assay technologies combined with expanding knowledge about G-protein-coupled receptors, nuclear receptors, ion channels, and enzymes make it possible to capture relative potency data in a large number of in vitro assays [8, 9]. Although not recommended as core assays in ICH safety pharmacology guidelines, results from

in vitro assays can inform sponsors of potential activities, and these results are valuable in improved design of in vivo safety pharmacology studies as well as interpretation of results from safety pharmacology and clinical studies.

The value of in vitro data in the overall safety risk assessment is greatest when used in conjunction with results from in vivo (including clinical) studies. In most cases, the drug concentration at its site of action cannot be measured in vivo, and therefore concentration levels of drug in plasma (often referred to as plasma exposure levels) are assumed to be proportional to the drug concentration at the site of action. Translation of the threshold and concentration–response information from in vitro assays to plasma exposure levels in vivo is influenced by many factors including distribution, metabolism and excretion, and plasma protein binding. In almost all cases, the threshold plasma exposure levels for an effect are greater in in vivo assays, and differences between in vitro and in vivo potencies vary among drugs. Finally, in the clinical setting, there may be reflexes and homeostatic mechanisms that dampen the response to the drug candidate. This is the case for drugs with vasodilator activity where the autonomic nervous system reflexes diminish the risk for treatment-related hypotension in patients. Ideally, the overall risk assessment will take into consideration both the direct actions of the drug candidate as well as the clinically relevant responses.

A general roadmap for guiding safety pharmacology investigations is provided in Table 1. These basic, sequential steps are consistent with recommendations in the ICH guidelines and intended to integrate safety pharmacology investigations into the overall development program. There are four categories: (1) identification of safety pharmacology activity, (2) characterization of the safety pharmacology activity, (3) context of the safety pharmacology activity with respect to patient safety, and (4) overall risk assessment. Successful navigation through these steps will facilitate interpretation of the safety pharmacology results for improved decision-making as well as improve communication of the safety assessment for drug development candidates.

4.1 Identification of Safety Pharmacology Activities

Safety pharmacology activity can be identified from many different sources during the drug discovery and development programs (see Table 1). In each of these cases, identification of the activity early enables design and interpretation of safety pharmacology investigations that significantly contribute to a better understanding of the implications of the activity and potential impact on patient safety. For example, if the drug candidate is from a class with a known safety liability, a head-to-head comparison with the reference drug will enable a sponsor to better understand the relative risk for their candidate. Opportunities to employ safety pharmacology studies to aid in lead optimization in drug discovery and to contribute in the interpretation of results in clinical studies should be considered.

Table 1 Roadmap for safety pharmacology investigations

I. Identification of safety pharmacology activities
a. Known safety liability from other drugs in the class
b. Results from selectivity screens in lead optimization
c. Results from core safety pharmacology studies
d. Activity identified in toxicology studies
e. Activity identified in clinical studies
II. Characterization of safety pharmacology activities
a. Parent versus metabolite
b. PK–PD (including variability)
c. Mechanism of action (mechanism based versus off target)
d. Relative potency (NOEL and dose–response relationship)
e. Comparison with positive and negative controls
f. Use of follow-up (Tier II) assays
III. Context of safety pharmacology activity and safety risk
a. Support conclusions with information of sensitivity and specificity for safety pharmacology assays used
b. Safety margin (potency relative to targeted therapeutic exposure)
c. Patient population
1. Other risk factors that influence safety
2. Impact of drug-drug interaction
3. Variation in exposure (hepatic or renal insufficiency)
IV. Overall risk assessment
a. Integrate safety pharmacology results with results from pharmacology, toxicology, and clinical studies
b. Compare risk with positive and negative control drugs with clinical experience
c. Evidence of risk – quantitate with respect to positive and negative control drugs with clinical experience
d. Update risk assessment as new information becomes available

The ability to detect unanticipated pharmacological activity of a drug candidate or chemical lead series can be a challenge because, in most cases, one only finds what they look for. How does one cast a wide enough net to reduce risk but not by consuming extraordinary resources? As discussed above, in vitro screening panels (ligand binding assays, enzyme assays) are effective tools for detecting off-target activities. This is a reasonable starting point with the understanding that only the activity of parent (not metabolites) will be detected. The core safety pharmacology assays recommended in ICH S7A and S7B are intended to be broad screens for unanticipated pharmacological activity but are not comprehensive for all potential liabilities. Therefore, lack of activity with a drug candidate in in vitro off-target screens and core safety pharmacology assays increases the probability that there will be no significant safety liabilities; however, this will be a function of the experimental design and quality of the assays used. When sponsors report lack of activity with their development candidate in safety pharmacology assays, they should also report the sensitivity of the assays used to support the conclusions of safety. In cases, where no activity was found in safety pharmacology assays but

there is activity evident later in development (e.g., in toxicology or clinical studies), revisiting data from the safety pharmacology assays can be very valuable for both understanding the safety risk as well as limitations of the safety pharmacology assays. This has been particularly true for assessing the risk of QT interval prolongation.

4.2 Characterization of Safety Pharmacology Activity

Gaining a further understanding of the safety pharmacology activity is critical for making a useful risk assessment. A careful examination of the pharmacokinetic/pharmacodynamic (PK–PD) relationship will help confirm that the observed effect is pharmacologically relevant and help to determine if the effect is a consequence of parent or metabolite. Determining the NOEL for the activity using plasma concentrations will enable predictions of exposure thresholds for safety in the clinical as well as estimating safety margins.

Knowledge of the mechanism of action for the safety pharmacology activity can be extremely valuable. This information can help (1) design more specific counter screens for optimizing an improved development candidate and (2) compare to marketed drug with the same mechanism and make better predictions about safety in humans. An example is torcetrapib where there were very modest blood pressure elevations in a subset of patients at the therapeutic dose. The mechanism for the blood pressure effect was associated with an increase in circulating aldosterone [10], which is a known cardiovascular risk factor and, therefore, may have contributed to increased mortality in the torcetrapib phase III program [11]. If the mechanism of the safety pharmacology activity is the same as the therapeutic mechanism, there is unlikely to be an opportunity for this class to be devoid of the safety pharmacology activity, and the sponsor will determine if the activity can be managed during clinical development.

The potential value of results from follow-up or Tier II safety pharmacology assays should be considered. This is particularly true in cases where a nonclinical assay can explore mechanisms more effectively and/or more economically than clinical studies. These studies are usually designed with a specific hypothesis, and, ideally, responses are compared to responses with a positive reference drug with a known clinical safety profile.

4.3 Context of Safety Pharmacology Activity and Safety Risk

Results from safety pharmacology studies require context in order to be incorporated into a scientifically based risk assessment. Documentation of assay sensitivity and specificity will help to determine the confidence that can be placed on the results from safety pharmacology assays. If the assay has relatively low

sensitivity, the potency estimates (NOEL) may be greater. Knowledge of targeted therapeutic exposure will enable estimation of safety margins. Note that for acute, direct effects from safety pharmacology studies, safety margins based upon C_{max} may be more relevant than when based upon AUC values.

The safety of a drug candidate can be influenced by the targeted patient population. If there is a risk for drug–drug interactions with the development candidate, are these patients likely to be on multiple medications? Are there underlying disease states which will put a subset of patients at greater risk for AEs? If primary routes of excretion are hepatic or renal, will elevated plasma exposures in patients with hepatic or renal insufficiency put patients at higher risk for adverse events?

4.4 Overall Risk Assessment

The data and information from the safety pharmacology studies are building blocks for developing an integrated risk assessment (see Sect. 5.2) which enables one to consider the balance between benefit (efficacy) and risk (tolerability and safety) for the drug candidate. While it is often unknown how observations from safety pharmacology studies will translate into the clinical setting, the availability of this information helps the development team become aware of potential risks so they can be managed in as safe a manner as possible.

There are many ways in which safety pharmacology can be valuable for sponsors, drug development teams, regulators, and investors. Value of the data may be immediate or realized after availability of toxicology or clinical findings in subsequent studies. One should consider safety pharmacology data and use follow-up studies throughout the development process to improve development decisions and ultimately support registration as well as post-marketing safety.

5 Maximize Safety Pharmacology Activities to Reduce Attrition

5.1 Consideration in Design of Safety Pharmacology Studies

The choice and design of safety pharmacology assays depends on the stage of development, how the results will be used, and the sponsor's risk tolerance. The assays recommended in the ICH guidelines are discussed in detail in several places [7, 12, 13]. In this chapter safety pharmacology is presented as an activity in the drug development tool box to facilitate progression of drug candidates through key decision and regulatory milestones and is not limited to the core assays described in the ICH S7A and S7B guidelines. The principles and recommended experimental considerations in ICH S7 provide a framework that can be adapted to the design of

all safety pharmacology experiments regardless of whether the results are solely for internal decision-making or to support regulatory packages.

5.1.1 Timing of Safety Pharmacology Studies

The guidelines recommend completing the core studies prior to initiating clinical studies; however, the strategy for types and when safety pharmacology studies are performed is case-by-case based upon many factors. Ideally, sponsors would like to reduce drug candidate attrition by identifying potential liabilities as early as possible in the drug discovery/development process. For this strategy, some safety pharmacology assays might be used to profile early chemical leads so that results of the studies contribute to lead optimization and candidate selection. Screening for inhibition of I_{Kr} (also referred to as the hERG assay) is an example of a safety pharmacology assay commonly used during lead optimization. Alternatively, there may be programs and situations where the sponsor is willing to accept the risk of having no safety pharmacology data until just prior to clinical studies. In this case the sponsor will assume risk of attrition at the preclinical development stage in return for saving discovery resources and keeping the lead optimization process less complicated. If an unacceptable safety pharmacology activity is discovered after selection of the drug candidate, the medicinal chemists can go back to lead optimization with knowledge of the liability in the series. In this case, knowing the mechanism of the safety pharmacology activity will enable use of more efficient screens to guide the lead optimization efforts. In most cases, a compromise between these two approaches is the best balance of resources and risk.

The focus of the ICH S7A guideline is on core assays for regulatory scrutiny. It is not necessary to closely follow guidelines for assays to be used for internal decision-making in discovery and early development. There may be cases where the sponsor has used safety pharmacology assays in development and, at the preIND stage, is faced with the prospect of repeating the safety pharmacology studies under conditions that more closely comply with guideline recommendations (specifically GLP). ICH S7A guideline states that when these studies are not performed according to GLP, the data quality and integrity in safety pharmacology studies should be ensured. This is a situation where positive controls, careful definition of exposure, and assay sensitivity will be required to support conclusions. An example is the in vitro I_{Kr} assay (ICH S7B), where having high-quality data is critical for lead optimization or candidate qualification. If repeating this study under GLP does not significantly improve the scientific creditability of the I_{Kr} assay results or the overall assessment of risk for QT interval prolongation, a sponsor should not have to repeat the study. It is noted that there have been regional differences in the need for GLP for core studies to be fully compliment with ICH S7A guideline and the burden of proof that the data are robust enough to meet objectives of the guideline will be the responsibility of the sponsor.

5.1.2 Study Design

Dose Levels, Formulation, and Route of Administration

The ICH guidelines consider safety pharmacology studies to be similar to toxicology studies where dose levels should be elevated until activity is observed or toxicity prevents pharmacological assessment at higher exposures. Margins of safety, even if large, may not be satisfactory to comply with this regulatory requirement if no activity is detected. If the maximum dose levels are limited by toxicity or unfavorable pharmacokinetics and the margins are small, the evidence for safety will not be robust. In these cases, it may be necessary to use a formulation that differs from the clinical formulation in order to achieve exposure at higher multiples of exposure. Ideally, plasma levels with the low dose level in the safety pharmacology study will be in the range of clinical intended exposures, the high dose level will show some pharmacodynamic effect or toxicity, and the middle dose level will have no effects of concern and be a comfortable multiple above the intended clinical exposure.

Recognizing that the objective of safety pharmacology experiments is to examine the safety of the test article, achieving adequate exposure to support conclusions is very important. The ICH S7 guidelines suggest that the clinical route of administration is preferred but also provide flexibility to use alternative routes in order to achieve exposure levels sufficient to support safety. It is more important to achieve appropriate exposure levels than to use clinical route of administration. This is particularly important when exposure by the clinical route is limited by maximal feasible exposure and/or saturation of exposure in the nonclinical safety pharmacology model. The dose levels and route of administration used in the safety pharmacology studies should be justified and achieve larger exposure levels than the targeted clinical exposure level.

Safety pharmacology studies are generally performed with single-dose administration (Sect. 2.5 in ICH S7A). The rationale is that one is looking for direct pharmacological effects of the test substance in the absence of histopathological changes. The acute, direct pharmacodynamic effect of a drug candidate is one dimension of the safety investigation and, according to ICH guidelines, appropriate for screening for unanticipated activity(ies). The limitations of conclusions from single-dose administration should be acknowledged in the integrated risk assessment (see Sect. 5.2).

There will be occasions where repeated administration in safety pharmacology studies is useful in assessing risk. One is when repeat dosing is necessary to get adequate exposure levels or a desire to get to achieve steady-state exposure levels in order to refine estimates of safety margins. Another situation where repeat dosing may be required is when the safety pharmacology study is investigating the pharmacodynamic consequences of an observation from a toxicology study. This type of follow-up study could be helpful in looking for consequences of toxicity observation and/or potential pharmacodynamic signals that might precede the

histopathological changes. This strategy is not described in the ICH guidelines but can be valuable in validating a pharmacodynamic "biomarker" that can be used to determine when to halt dosing before there are more serious consequences to function or structure. The scientific rationale for a repeat-dose protocol and confidence in results from these studies should be defendable and clearly communicated in regulatory submissions.

5.1.3 Documenting Drug Exposure

Knowledge of the drug exposure–response relationship is critical in supporting the risk assessment from safety pharmacology data. Differences in metabolism and pharmacokinetics between the nonclinical models and humans render dose–response relationships alone in the risk assessment to be of lesser value. Using plasma levels of the drug candidate to document exposure from the nonclinical preparation where the pharmacodynamic response was observed is ideal. In some case, there is concern that acquiring blood samples from conscious, unrestrained animals on study will compromise the quality of the pharmacodynamic measurements. The sponsor should consider the benefit to risk of collecting plasma samples in the same study. A default strategy is to use a separate group of animals to obtain pharmacokinetic data or estimate exposure from a study with similar design (such as toxicokinetic data on day 1 of a toxicology study at the dose levels and in the same species).

In the majority of cases, the magnitude of the pharmacodynamic effect is related to exposure (concentration) in a semi-log manner. Defining this relationship with at least 2 levels of response is extremely valuable because (1) it will strengthen the conclusions that the response is treatment related (i.e., not false positive) and (2) will facilitate estimation of threshold response (for estimating margins over anticipated clinically relevant exposures) and relative potency to reference drugs.

Ideally, steady-state plasma concentration should be used to determine the concentration–response relationship, but it is very difficult to achieve steady-state levels with single-dose safety pharmacology studies. At steady state, the assumption that plasma concentration reflects drug concentration at pharmacological target is more likely to be valid since distribution of drug should not be changing with time. In the ICH guideline S7A, it is recommended that pharmacodynamic effect should be measured at maximum plasma concentration (C_{max}). However, for some drugs, the time for distribution of drug to the pharmacological site of action is longer than the plasma T_{max} (time to C_{max}). Two examples are (1) drugs with a site of action in the central nervous system (CNS) where CNS concentration is a function of the rate which the drug crosses the blood–brain barrier and whether it is a substrate for transport systems that remove chemicals from the CNS and (2) drugs that are very lipophilic, particularly if it is basic and has a high volume of distribution causing it to accumulate in tissues over time and achieve a greater concentration in target organ compared to plasma. The differential time course for distribution of drug in plasma versus target organ is referred to as hysteresis.

When there is hysteresis, there is risk that the potency will be under estimated at T_{max} for plasma levels (i.e., plasma C_{max}, concentration is greater than that at the pharmacological site of action). It was the goal of a recent workshop to model PK–PD relationships from safety pharmacology studies to better predict relative potencies in humans (see [14]). One approach to achieve better concentration–response (PK–PD) relationship for drugs with shorter half lives is to administer test drug via intravenous infusions.

Plasma protein binding can significantly reduce the "available" concentration of drug at molecular site of action and the in vivo potency for the effect. While it may be a fortuitous characteristic to reduce potencies of an undesired activity, one should also consider the likelihood that plasma protein binding will also limit "available" concentration for the desired pharmacological activity. Plasma protein binding is usually expressed as percent bound and some advocate using this value to "adjust" potencies in the risk assessment [15]. Unfortunately, it is not that simple because the availability of drug for potential pharmacodynamic activity is the equilibrium between drug binding to plasma protein and the molecular target [16] and not merely percent not bound. Percent binding does not capture this dynamic situation adequately. The most practical approach is to compare protein binding in plasma from the species used for the in vivo assay and humans, and if reasonably similar, use in vivo potencies (PK–PD relationship above) to associate risk with exposure in humans. Positive controls and reference drugs are also useful in this regard.

5.1.4 Metabolites

The overall safety of a drug is dependent not only on the safety of the parent drug but also circulating metabolites. Therefore, consideration of potential human metabolites in planning safety pharmacology studies is important. In ICH S7A: "Generally, any parent compound and its major metabolite(s) that achieve systemic exposure or are expected to reach the systemic circulation in humans should be evaluated in safety pharmacology studies." Since safety pharmacology studies are typically done before there is information on metabolism in humans, this can be an imperfect situation. Ideally, the metabolite profile in humans and the safety pharmacology models are sufficiently similar so that when dosing with parent drug, exposure to metabolite(s) is sufficient to profile them for unanticipated pharmacological activity. When there is a presumed major metabolite, it would be useful to measure plasma concentrations of the metabolite in order to document its safety.

What constitutes a major metabolite has been a topic of debate [17, 18] and is the topic of a recent FDA guidance [19]. If a human metabolite is discovered after the drug candidate has progressed into clinical studies, it may be prudent to consider whether exposure in the safety pharmacology studies was sufficient to support safety. In some cases, the clinical safety profile is adequate and additional safety pharmacology studies are not necessary. All of this information should be included in the integrated risk assessment (see below). These decisions concerning what

metabolites to measure and whether specific metabolite testing is appropriate should be based upon the strength of the scientific argument and an assessment of the resource/risk ratio of not having the information.

5.1.5 Nonclinical Experimental Preparations

The selection of nonclinical assays and data analysis to support the safety of a new drug candidate should be scientifically based. While the guidelines provide direction and lay out expectations of the regulatory agencies, limiting the studies to those recommended in the guidelines may limit the strength of the safety package. Deviations from the guidelines should be acknowledged and explained to help regulators clearly understand the strategy.

5.1.6 In Vitro and In Vivo Assays

In vitro assays are very useful for elucidating mechanisms and defining direct effects of drug candidates although it is often difficult to translate potencies from in vitro assay results alone to the clinical setting. In vitro and in vivo assays are complementary, and extending the in vitro results into an in vivo model is a good way to investigate the potential consequences of off-target activity at the organ level. For example, if there is unanticipated activity at the alpha adrenergic receptor in an in vitro assay, potential consequences on cardiovascular and central nervous system function can be better defined with in vivo studies designed to determine at what exposure level is this a safety issue. The in vivo assays can also be used to interrogate potential contribution of metabolites to safety pharmacology activity.

5.1.7 Anesthetized Versus Conscious In Vivo Preparations

The guidelines recommend use of conscious, unrestrained animals and the clinical route of administration for safety pharmacology assays. While the premise is that these assays conditions are more “physiological” and resemble the clinical setting, they may not be the most sensitive conditions for detecting and characterizing pharmacological activity. Most cardiovascular safety pharmacology studies are performed in conscious, unrestrained subjects using telemetry devices to capture data. Unlike subjects in a phase I clinical study, conscious animals in the safety pharmacology study are not necessarily calm and there can be large changes in baseline blood pressure, heart rate, and ECG values. This is particularly true for nonhuman primates where heart rates can range from 80 to 250 beat/min during an experiment. Anesthetized preparation has advantages of being able to interrogate acute pharmacological effects of the drug candidate with a stable baseline and controlled levels of plasma exposures (e.g., with intravenous infusions). If the drug candidate can be formulated appropriately, the peak plasma exposures with

intravenous infusions are often greater than can be achieved by the oral route and, with a stepwise increase in infusion dose levels, a full dose (exposure)–response study can be achieved in each experiment. This assumes that a stable plane of anesthesia is maintained during the study and the type and influence of anesthesia on baseline values and autonomic tone are considered in the interpretation of the data.

Given this information, anesthetized preparations can be used for cardiovascular assessments in two different ways. First, anesthetized preparations can be to screen for cardiovascular activity as part of the candidate selection process. The experiment can be used to interrogate findings from the off-target screens and assess risk of cardiovascular effects. Results from the conscious preparation will be used in combination with results from the anesthetized preparation to gain advantages of both models and enhance the risk assessment. Alternatively, one can use the anesthetized assay as a follow-up assay when needed to further characterize cardiovascular activity of the drug candidate when there are findings in studies with conscious subjects. For example, reflex tachycardia often accompanies vasodilator responses, and use of an anesthetized preparation will help determine if the increase in heart rate is a direct effect of the drug candidate or a consequence of vasodilatation. Also changes in heart rate due to general behavior or other noncardiovascular effects can confound interpretation of risk for QT/QTc interval prolongation, and the heart rate effects will be reduced in anesthetized preparation.

5.1.8 Disease Animal Models

Drugs are used in subjects with disease and they have a variety of risk factors that can influence their safety. The rationale for use of animals without disease in safety pharmacology and toxicology studies, as well as normal volunteers in Phase I clinical trials, is that these models provide a more homogenous, reproducible setting for detecting and characterizing the pharmacological/toxicological activities of the drug candidate. Clinical safety in patients for marketing registration is demonstrated in a large number of patients and continues to be monitored after marketing approval so that even more safety experience is achieved. It is impractical to assume that an animal model of disease can be used to define safety in the broad and varied population of targeted patients; however, there are cases where testing drug candidates in disease models can add value to the risk assessment and reduce risk for attrition in late clinical development. Data from a disease model is most effective when results are compared to a reference drug. For example, to evaluate the risk of a drug candidate to cause bronchoconstriction in an asthma model, results should be reported in the context of the positive control, i.e., drug candidate at dose levels X-fold above the therapeutic level does not cause bronchoconstriction in a model where drug Y (positive control) causes bronchoconstriction at a dose level known to cause this effect in patients, or like drug Y positive control, which causes bronchoconstriction in asthmatic patients, the drug candidate is Z-fold less potent in causing bronchoconstriction as drug Y. It is important to interpret and report the results in the context of the conditions of the experimental disease model with an

understanding that the results may not apply to all types of patients with the disease. Following recommendations in the guidelines, the choice and sensitivity of the disease model should be justified in the regulatory submission.

5.1.9 Sensitivity of Safety Pharmacology Assays

There are no recommendations in the ICH guidelines for the degree of sensitivity of the safety pharmacology assays; however, the guidelines do recommend that the sensitivity of the assays be defined. In this manner, results can be interpreted in the context of assay sensitivity which is particularly important when the findings indicate there is no activity with the drug candidate at dose levels tested. Given the small number of subjects used in each safety pharmacology study, it is impractical to achieve sensitivity at clinically relevant dose levels adequate to exclude all risk in patients. This means the relative potency for the effect will be lower in a safety pharmacology assay with low sensitivity, but this can be handled by comparing the relative potency of the drug candidate with that of a positive control drug that has the effect in humans. For example, one might conclude that the drug candidate does not prolong the QTc interval in a safety pharmacology model where the threshold for drug X (positive control) increases the QT/QTc interval at similar exposure levels.

In defining sensitivity and validating safety pharmacology models, it is important to evaluate negative controls and define thresholds for positive controls. Merely showing that a positive control elicits a large response at a high dose level does not adequately support the lack of activity for a drug candidate. Therefore dose (concentration)–response curves should be obtained with positive and negative controls to best support conclusions and comply with the regulatory recommendations.

5.2 The Integrated Risk Assessment and Communication of Results

No drug candidate is completely devoid of potential safety risks. The objective is to determine the degree and types of risk and put them in the context of intended clinical use, targeted patient population, and safety of available drugs. Central to overall strategic decisions in drug development is the benefit to risk ratio. An understanding of the potential risks for safety and adverse events contributes to decisions concerning development and investments and risk mitigation strategies.

Results from safety pharmacology studies require context in order to translate the information into a risk assessment (Table 1). One should consider the following: Is the activity an adverse effect by itself, or is it one of a combination of risks factor for adverse outcomes? Is the potency of the safety pharmacology finding

close to the predicted therapeutic exposure? Is the activity reversible? Other characteristics of the drug are very important. Will the drug be used chronically or acutely? If the safety margins are small, is there a risk for elevated plasma levels due to variable absorption, drug–drug interactions, or in patients with renal or hepatic insufficiency? Are there other patient subpopulations, health conditions, or activities of other drugs used in combination with the drug candidate that will enhance the risk of revealing an adverse effect?

In the ICH S7B guideline, it is recommended that the sponsor develops an integrated risk assessment for QT interval prolongation and communicates overall conclusion in terms of "evidence of risk." This is an effective tool for communicating results from all types of safety pharmacology studies. Following recommendations in the ICH guideline, all available data and information should be considered in the integrated assessment (Table 1). This is a scientific-based document and therefore facts and data should be used to support conclusions. It is also useful to point out what is not known and how the related risks will be mitigated or managed safely in clinical studies. The risk assessment is a living document and should be reevaluated as additional data become available throughout development. In preparing the document, it is important to consider the reader (e.g., internal decision-making body, regulators, or investors) and include adequate background information and descriptions of the results to avoid misinterpretation. When results from noncore studies (follow-up studies) are included, it is recommended to design the study with a testable hypothesis and consider scenarios as well as their impact on decisions in advance. Avoid doing experiments to "see what happens" or with the sole expectation that there will be no adverse findings.

An integrated risk assessment is an important opportunity to lay out the scientific evidence to support the safety of the drug candidate. By not doing this, sponsors leave reviewers to interpret the results and may come to inappropriate conclusions about safety. The source documents (study reports) often only describe the experimental protocol and result and seldom include background, adequate justification for protocols, or interpretation of results. This is particularly true when reports from contract research organizations (CROs) are used because the CRO usually does not have a full perspective of the program. The integrated risk assessment should be included in the IND and NDA documents as well as investigator brochures.

6 Future Opportunities for Safety Pharmacology

Safety pharmacology is a developing discipline with several ongoing initiatives to improve the quality of information and reduce use of resources [20, 21]. A few examples are listed. With the availability of jackets for collecting cardiovascular and respiratory data in dogs and monkeys, safety pharmacology endpoints can be captured in toxicology studies [22, 23]. Human stem cell-derived cardiomyocytes are being investigated as a new model for safety pharmacology studies [24]. There is also an ongoing collaboration between Pharma and the FDA to consider whether nonclinical

studies can be sufficient to reduce the recommendations for a clinical thorough QT/QTc study [25].

7 Concluding Remarks

The primary goal of safety pharmacology studies is to define risks for attrition so that clinical candidates with lower risk of attrition are advanced or with knowledge of risks for attrition, they can be appropriately managed during development. The value of safety pharmacology studies in contributing to drug discovery and development strategy and decisions is a direct function of the scientific quality of the data combined with accurate interpretation and effective translation of results into a risk assessment. Without information on the sensitivity and potencies of reference drugs in the assay, results with the drug are incomplete. When no activity is found in an assay, sponsors need to be able to defend the conclusions. Likewise, if results and information from all sources are not included in an integrated risk assessment, decisions concerning risk of attrition will be less informed.

References

1. Kola I, Landis J (2004) Can the pharmaceutical industry reduce attrition rates? Nat Rev Drug Discov 3:711
2. Paul SM, Mytelka DS, Dunwiddie CT, Persinger CC, Munos BH, Lindborg SR, Schacht AL (2010) How to improve R&D productivity: the pharmaceutical industry's grand challenge. Nat Rev Drug Discov 9:203
3. Pammolli F, Magazzini L, Riccaboni M (2011) The productivity crisis in pharmaceutical R&D. Nat Rev Drug Discov 10:428
4. Mahajan R, Gupta K (2010) Food and drug administration's critical path initiative and innovations in drug development paradigm: challenges, progress, and controversies. J Pharm Bioallied Sci 2:307
5. Zbinden G (1979) Pharmacological methods in toxicology. Pergamon, Elmsford
6. Bednar MM, Harrigan EP, Anziano RJ, Camm AJ, Ruskin JN (2001) The QT interval. Prog Cardiovasc Dis 43:1
7. Koerner JE, Siegl PKS (2013) Safety pharmacology: guidelines S7A and S7B. In: van der Laan JW, DeGeorge JJ (eds) Global approach in safety testing, aaps advances in the pharmaceutical sciences series, vol 5. Springer, p 243
8. Whitebread S, Hamon J, Bojanic D, Urban L (2005) Keynote review: in vitro safety pharmacology profiling: an essential tool for successful drug development. Drug Discov Today 10: 1421
9. Bowes J, Brown AJ, Hamon J, Jarolimek W, Sridhar A, Waldron G, Whitebread S (2012) Reducing safety-related drug attrition: the use of in vitro pharmacological profiling. Nature Reviews Drug Discovery 11:909
10. Forrest MJ, Bloomfield D, Briscoe RJ, Brown PN, Cumiskey A-M, Ehrhart J et al (2008) Torcetrapib-induced blood pressure elevation is independent of CETP inhibition and is accompanied by increased circulating levels of aldosterone. Br J Pharmacol 154:1465
11. Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJP, Komajda M et al (2007) Effects of torcetrapib in patients at high risk for coronary events. N Engl J Med 357:2109

12. Bass AS, Siegl PK, Gintant GA, Murphy D, Porsolt R (2008) In Gad SC (ed) Preclinical development handbook: toxicology. Wiley, Chicester, Chap. 18, p 611
13. Gad SC (2012) Safety pharmacology in pharmaceutical development: approval and post-marketing surveillance. CRC, Boca Raton
14. Cavero I (2007) Using pharmacokinetic/pharmacodynamic modelling in safety pharmacology to better define safety margins: a regional workshop of the Safety Pharmacology Society. Expert Opin Drug Saf 6:465
15. Redfern WS, Carlsson L, Davis AS, Lynch WG, MacKenzie I, Palethorpe S, Siegl PKS, Strang I, Sullivan AT, Wallis R, Camm AJ, Hammond TG (2003) Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development. Cardiovasc Res 58:32
16. Smith DA, Di L, Kerns EH (2010) The effect of plasma protein binding on in vivo efficacy: misconceptions in drug discovery. Nat Rev Drug Discov 9:929
17. Baillie TA, Cayen MN, Fouda H, Gerson RJ, Green JD, Grossman SJ, Klunk LJ, LeBlanc BA, Perkins DG, Shipley LA (2002) Drug metabolites in safety testing. Toxicol Appl Pharmacol 182(3):188–196
18. Hastings KL, El-Hage J, Jacobs A, Leighton J, Morse D, Osterberg R (2003) Drug metabolites in safety testing. Toxicol Appl Pharmacol 190(1):91–92
19. FDA Guidance (2008) Safety testing of drug metabolites. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079266.pdf
20. Cavero I (2013) 2012 Annual Meeting of the Safety Pharmacology Society: spotlight on targeted oncology medicines. Expert Opin Drug Saf 12:589–603
21. Pugsley MK, Curtis MJ (2012) Methodological innovations expand the safety pharmacology horizon. J Pharmacol Toxicol Methods 66(2):59–62
22. Guth BD, Bass AS, Briscoe RJ, Chivers S, Siegl P, Valentin J-P (2009) Comparison of electrocardiographic analysis for risk of QT interval prolongation using safety pharmacology and toxicological studies. J Pharmacol Toxicol Methods 60:107
23. Redfern WS, Ewart LC, Lainée P, Pinches M, Robinson S, Valentin J-P (2013) Functional assessments in repeat-dose toxicity studies: the art of the possible. Toxicol Res http://pubs.rsc.org/en/content/articlelanding/2013/tx/c3tx20093k
24. Jonsson MK, Vos MA, Mirams GR, Duker G, Sartipy P, de Boer TP, van Veen TA (2012) Application of human stem cell-derived cardiomyocytes in safety pharmacology requires caution beyond hERG. J Mol Cell Cardiol 52:998
25. Trepakova ES, Koerner J, Pettit SD, Valentin JP; HESI Pro-Arrhythmia Committee (2009) A HESI consortium approach to assess the human predictive value of non-clinical repolarization assays. J Pharmacol Toxicol Methods 60:45

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