3 Role of ADME Studies in Selecting Drug Candidates: Dependence of ADME Parameters on Physicochemical Properties

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3.1 SUMMARY

Drug discovery is a time-consuming and expensive endeavor fraught with many glaring failures in clinical trials. Optimization of drug absorption, distribution, metabolism, and excretion (ADME) parameters continues to play an important role to ensure that the exposure is sufficient to achieve proof of concept, and ultimately efficacy, safely in clinical trials to address unmet medical need. This chapter provides an overview of various *in vitro* and *in vivo* ADME studies commonly employed in drug discovery. Emphasis will be placed on the dependence of ADME parameters on physicochemical properties. The literature has been reviewed to highlight key relationships. However, literature reviews mainly focus on retrospective analyses. Therefore, examples are

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included to illustrate how these principles can be embedded in drug discovery and used in a prospective drug design fashion with an emphasis on finding the physicochemical "sweet spot" that balances various properties. *In silico* (ADME) models should be an integral part of design as well and the value of various models has been illustrated.

3.2 THE ROLE OF ADME STUDIES IN DRUG DISCOVERY

Determining drug ADME properties of drug candidates early on in the drug discovery process is critical for achieving drugs with optimal properties and, in particular, an acceptable dose and dosing regimen. Many people refer to the data presented in Fig. 3.1—the reasons for attrition in development in 1991 and 2000—to highlight the impact of early ADME studies and their positive impact on a significant reduction in attrition due to pharmacokinetic (PK) reasons. In the 1980s, inappropriate ADME characteristics were a leading cause of attrition in drug development (about 40% of drugs failed due to drug metabolism and PK reasons) and this was reduced to $<10\%$ in the 1990s [1]. The latter was confirmed by another analysis of drug development project terminations from 1992 until 2002 with ADME being cited as the reason for attrition in 14%, 17%, and 4% in phase I, II, and III, respectively [2]. Although this trend is undoubtedly real, it is important not to overinterpret these data and assume that ADME sciences are sufficiently mature. First, there were many anti-infectives in development in the 1980s with many of them failing due to poor PK and contributing to the statistics presented in Fig. 3.1. If anti-infectives are taken out of the equation, the reduction in attrition due to ADME reasons is less pronounced; the attrition due to PK reasons was 39% from 1964 until 1985, but this was reduced to 7% if anti-infectives

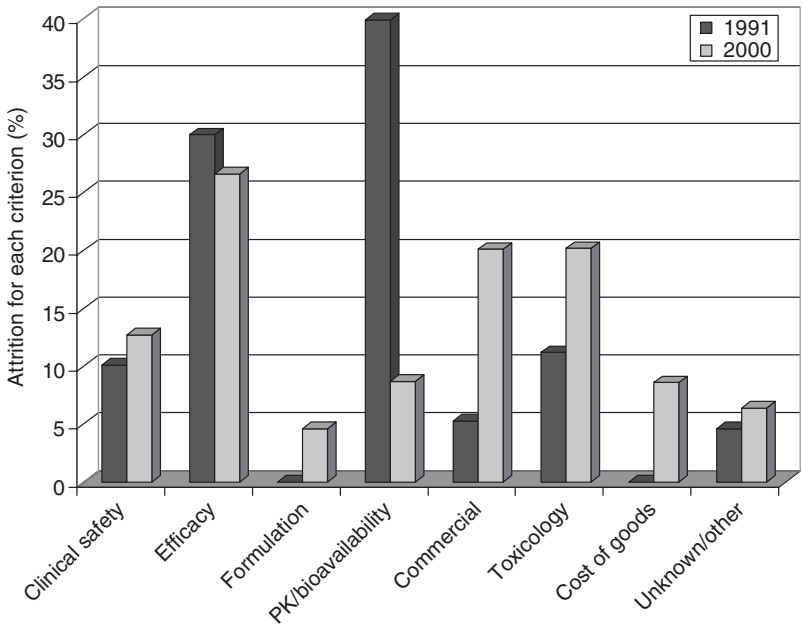


Figure 3.1 Reasons for attrition of drugs in development. *Source*: Data from Ref. 1.

were excluded [3]. Second, many compounds fail in development because they fail to illicit efficacy. Indeed, a key concern in the pharmaceutical industry is the very high attrition in phase 2 (>60% is quite common among the leading pharmaceutical companies and it is getting worse) and this is largely due to lack of efficacy or insufficient efficacy compared with standard of care [4,5]. However, in some cases, the lack of efficacy may be due to insufficient exposure and it is not inconceivable that efficacy may have been achieved if the clearance had been lower or absorption better. The latter is particularly worrisome because the clinical validity of the target may not have been properly assessed and, therefore, the decision to nominate a backup compound or not cannot be made in a reliable manner. Indeed, 12 of 44 phase 2 programs failed at Pfizer because no downstream pharmacological effect was observed in human and confidence that sufficient exposure was achieved to test the mechanism was low [6]. Third, some of the attrition due to toxicity may be metabolically mediated and, therefore, a greater understanding of the metabolic fate of drug candidates could influence survival in drug development. Moreover, the regulatory standards keep increasing with an increasing demand for ADME studies and a reduced tolerance for ADME-related issues such as drug–drug interaction and reactive metabolite formation. Thus, although the trend is very encouraging, it is critically important to continue to advocate early incorporation of ADME properties in the complex multiparameter optimization process in drug discovery and do so on an equal footing with potency, selectivity, toxicity, and so on. The important role of ADME properties is nicely illustrated by the "*stringent selection criteria*" presented by AstraZeneca in 2011 [7] and reproduced in Table 3.1 with the parameters having a direct bearing on ADME highlighted.

In vitro and *in vivo* ADME studies serve multiple roles in drug discovery:

- Determination of *in vitro* ADME parameters using cell lines and subcellular fractions from rodents, dogs, or monkeys to identify the best compounds for preclinical PK studies or *in vivo* proof of concept studies.
- Determination of plasma or tissue exposure to prepare for or to support interpretation of preclinical efficacy and toxicity studies.
- Determination of *in vitro* ADME parameters using human cell lines and subcellular fractions and *in vivo* ADME parameters from studies in rodents, dogs, or monkeys to predict human PK parameters and the propensity for drug–drug interaction.

TABLE 3.1 Stringent Selection Criteria Presented by AstraZeneca in 2011 [7]

Right Target Engagement	Link Between Target/Disease
	Predictive Biomarkers
Right tissue exposure	*Bioavailability and tissue exposure*
	Human PK/PD prediction
Right safety	Differentiating safety
	Reactive metabolites
Right patients	Scientific evidence in lead indication
	Stratification of patient population
Right commercial	Differentiated value proposition
	Embedded payer perspective

- Determination of the anticipated human efficacious dose using a combination of predicted human PK parameters and preclinical pharmacodynamic (PD) and/or efficacy studies.

Considerable progress has been made with the suitable incorporation of preclinical *in vitro* and *in vivo* ADME studies, but understanding the ADME structure-activity relationship (SAR) and the ability to effectively manipulate it in drug discovery is still problematic. Indeed, in many cases, it is easier to optimize potency and selectivity than the ADME properties, which provides further impetus to incorporate ADME in drug design early on. The following DMPK-centric strategies can have a positive impact on identification of compounds with superior properties and result in a reduction in the time spent in drug discovery:

- (continued) emphasis on physicochemical properties in drug design
- use of *in silico* ADME models to enable assessment of ADME properties before synthesis
- early incorporation of *in vitro* and *in vivo* ADME studies in drug discovery to identify compounds with optimal preclinical and ultimately clinical ADME attributes as well as potency, selectivity, toxicity, and so on; *in vitro* ADME data should be considered right after identification of hits from the biochemical high throughput screen (HTS) to facilitate identification of chemical series that are easier to optimize
- integration of all preclinical *in vitro* and *in vivo* data to improve the confidence in prediction of the human PK
- exposure–effect modeling with appropriate PD biomarkers in preclinical models to get a quantitative understanding of target and pathway modulation and the anticipated human exposure for sufficient target coverage and, subsequently, efficacy in humans; combination of these data with anticipated human PK will provide the expected efficacious dose
- detailed analysis of clinical PK and PD data to influence design of backup compounds

Interestingly, some have questioned the need for substantial ADME involvement in early drug discovery if the target is both clinically and preclinically unvalidated. Although it is understandable that certain ADME properties [such as reversible or time-dependent cytochrome P450 (CYP) inhibition] do not need to be considered if achieving preclinical proof of concept in an appropriate animal model is the main objective, having sufficient exposure at a reasonable dose will still require substantial involvement of ADME scientists.

In this chapter, a number of strategies have been outlined and examples included to illustrate the importance of ADME in drug discovery. Particular attention has been paid to physicochemical parameters because manipulation of these parameters is something medicinal chemists can relate to easily and, moreover, these parameters have a direct bearing on various ADME attributes. The ultimate goal is to find the right balance of physicochemical properties because some parameters have an orthogonal dependence on physiochemical parameters. For example, a low log D value may adversely influence absorption, but a high log D value may lead to poor solubility and, hence, poor

absorption and poor metabolic stability. Thus, proper incorporation and balancing of physiochemical parameters will have a positive impact on various ADME parameters and, therefore, increases the probability of success in drug discovery and development, although it does not guarantee it.

3.3 DRUG DESIGN AND THE IMPORTANCE OF PHYSICOCHEMICAL PROPERTIES: IS THERE LIFE BEYOND THE "RULE OF FIVE"?

The first thorough analysis of the impact of physicochemical parameters was performed by Christopher Lipinski at Pfizer in the late 1990s [8]. Lipinski proposed the "rule of five" and he argued that poor absorption is more likely if

- log $P > 5$
- molecular weight (MW) > 500
- number of hydrogen bond donors (HBDs) > 5
- number of hydrogen bond acceptors (HBAs) > 10

Fortunately, all these parameters can be calculated easily and incorporated in compound design. A recent analysis (Table 3.2) has shown that phase 1 oral drugs have a considerably higher MW and more HBAs and rotatable bonds than marketed oral drugs, which suggests that "rule of five" violations are associated with higher attrition in drug development. The latter has been confirmed by an independent analysis of a large corporate database showing that relatively speaking a higher percentage of compounds with "rule of five" violations attrite for PK reasons (Hop, unpublished data). In addition, an analysis of a database of oral drugs and the ChEMBL database of "hits" indicated that the mean MWs of drugs and "hits" are 333 and 430 Da, respectively, and the mean log P values are 2.5 and 3.5, respectively [9]. On the other hand, the chemical complexity of oral drugs launched between 1983 and 2002 versus those launched before 1983 has increased with a significant increase in MW and the number of HBAs and rotatable bonds (Table 3.2). The latter is not surprising because the introduction of high throughput screening has increased the emphasis on potency and it has been shown that potency increases with MW and log P in a statistically significant manner

TABLE 3.2 Average Physicochemical Properties of Oral Drugs in Phase I and Marketed Oral Drugs in Comparison with Lipinski's "Rule of Five"

	Phase 1 Oral Drugs	Marketed Oral Drugs	Oral Drugs Launched Pre-1983	Oral Drugs Launched 1983–2002	Lipinski's Rule of Five
Molecular weight (Da)	423	337	331	377	≤500
*c*log P	2.6	2.5	2.3	2.5	≤5
*c*log $D_{7.4}$	1.3	1.0	—	—	—
Hydrogen bond donors	2.5	2.1	1.8	1.8	≤5
Hydrogen bond acceptors	6.4	4.9	3.0	3.7	≤10
Rotatable bonds	7.8	5.9	5.0	6.4	—
Rings	—	—	2.6	2.9	

Source: Data from Wenlock *et al*. [11] and Leeson and Davis [12].

[9]. Nevertheless, the average properties of drugs launched between 1983 and 2002 are well within Lipinski's "rule of five." Analysis of a data set of 108 compounds with clinical data provided by multiple companies corroborates the trend toward greater molecular complexity and lipophilicity with an average MW of 443 Da (range: 171 to 725 Da) and an average log *P* of 3.75 (range: −5.95 to 12.1) [10]. Unfortunately, it is not known which of these 108 drug candidates—if any—were approved as new drug applications (NDAs).

Most of these analyses refer to clinical candidates nominated before Lipinski's "rule of five" was presented and, therefore, may not reflect current practices and the extent of incorporation of the "rule of five" and associated principles in drug design. Leeson and St-Galley [13] performed a comprehensive analysis of compounds patented by large pharmaceutical companies between 2000 and 2005, and 2006 and 2010, which could potentially reflect the degree of incorporation of the "rule of five" in compound design as it is practiced at this moment. Several interesting conclusions can be drawn.

1. The authors comment on the influence of the "organizational factor" on physicochemical parameters. The differences in *c*log *P* and MW across companies are striking with a one log unit difference in *c*log *P* between Wyeth (average *c*log *P* 4.5) and Pfizer and AstraZeneca (average *c*log *P* 3.5) and 60-Da difference in MW between Boehringer Ingelheim (average MW 495 Da) and Pfizer and Vertex (average MW 435 Da). Moreover, large organizational differences in *c*log *P* and MW are maintained if only shared targets are taken into consideration. For example, data have been presented for CCR5 antagonists showing a 2 log unit difference in mean log *P* and 100-Da difference in mean MW between leads from AstraZeneca, GlaxoSmithKline, Merck, and Pfizer [14]. Organizational differences were also obtained for other physicochemical properties such as the number of HBAs and HBDs, the number of rotatable bonds, and the number of sp^3 carbon atoms. An in-house analysis of the physicochemical properties of both the Genentech and Roche compound libraries showed significant differences with the median MW and *c*log *P* about 50 Da and close to 0.5 log unit, respectively, higher in the Genentech library than the Roche library. It is likely that the composition of the library has a direct bearing on the properties of the leads and ultimately drug candidates, although fragment-based approaches could have a positive influence, but its full impact may not yet be visible.
2. The mean *c*log *P* values decreased for many companied when comparing data from 2000 to 2005 and 2006 to 2010. Statistically significant reductions were noticed for Takeda, Lilly, Schering-Plough, and AstraZeneca. In contrast, the mean MW did not decrease significantly for most companies and even increased for some companies.
3. In agreement with observations by Morphy [15] and Paolini *et al*. [16], significant differences in *c*log *P* and MW were observed across targets, but in many cases, the mean values still did not violate the "rule of five." For example, for oral central nervous system (CNS) drugs, the "cutoffs" for parameters such as MW, number of HBAs and rotatable bonds, and polar surface area (PSA) have to be reduced to ensure that the drug can cross the blood–brain barrier [12,17] and this is illustrated in Table 3.3. This chapter focuses on oral drugs, but it is worth noting that different criteria apply for non-oral routes of administration, such as intravenous, topical, and inhaled.

TABLE 3.3 "Pajouhseh's Rules" for CNS Compounds

Molecular weight (Da)	<450
*c*log *P*	<5
Hydrogen bond donors	<3
Hydrogen bond acceptors	<7
Rotatable bonds	<8
PSA (Å^2)	60–90
pK_a	7.5–10.5

Source: Data from Pajouhesh and Lenz [17].

Several companies have confirmed that attrition of compounds breaking the "rule of five" is higher than those abiding by the "rule of five." Price *et al*. [18] have shown that physicochemical properties are not only important for superior ADME and pharmaceutical properties, there is also a statistically significant relationship with attrition due to toxicity. Specifically, they showed that attrition due to toxicity is about six- to seven-fold higher if *c*log $P > 3$ *and* topological polar surface area (TPSA) $< 75\,\text{Å}^2$ versus *c*log $P < 3$ *and* TPSA $> 75\,\text{Å}^2$. Although not confirmed, this could be partially due to increased off-target activity of these compounds. Indeed, Gleeson *et al*. [9] have shown that the number of off-target hits increases with increasing log *P* and MW. Alternatively, lipophilic compounds with *c*log $P > 3$ *and* TPSA $< 75\,\text{Å}^2$ could be less metabolically stable and (reactive) metabolites could contribute to toxicity.

Although most drug discovery scientists recognize the importance of good physicochemical properties, the degree of incorporation in drug design still varies and many claim that their target (e.g., disruption of protein–protein interaction) necessitates exploration of chemical space beyond traditional drug-like chemical space [19]. Indeed, very successful drugs that violate the "rule of five," such as atorvastatin, ritonavir, alendronic acid, and montelukast, are used as examples to justify their decisions. Although it is beyond doubt that successful drugs exist beyond the "rule of five," it is worthwhile to look at the attributes of some of these compounds in relation to their physicochemical properties. An exhaustive analysis is beyond the scope of this chapter, but a more detailed look at several examples from an internal analysis performed in 2006 provides valuable context; the structures of the drugs in question are presented in Fig. 3.2.

3.3.1 Alendronic Acid

Alendronic acid—a synthetic analog of pyrophosphate—has seven HBDs, which is a potential liability. Indeed, absorption is quite low with a bioavailability of about 1% and there is a substantial effect of food and beverages (in particular those rich in multivalent cations such as calcium) on absorption. However, alendronic acid is taken up in the bone—the site of action—and has a half-life of >10 years, which reflects the rate of bone turnover rendering the poor bioavailability less relevant.

3.3.2 Atorvastatin

The MW of the cholesterol-lowering drug atorvastatin is 558.6 Da and this violates the MW "limit" of 500 Da by 12%. The solubility of the calcium salt is very low

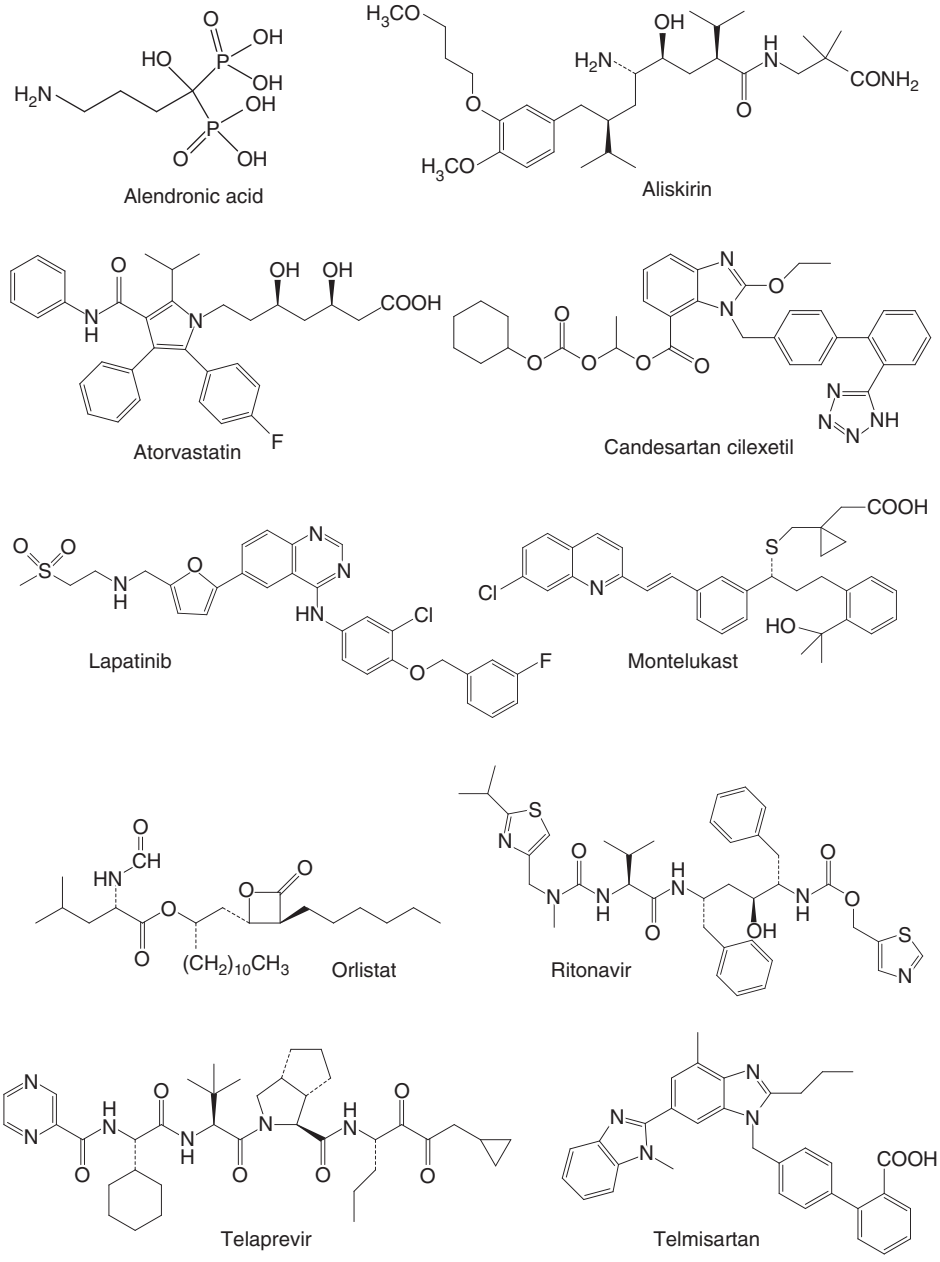


Figure 3.2 Structures of drugs referred to in the text.

and the intestinal and hepatic first-pass extraction ratios are high, 0.76 and 0.42, respectively [20]. Not surprisingly, the systemic exposure of the acid form of atorvastatin is relatively low and that may be perceived as a flaw. However, the acid form of atorvastatin is taken up in the liver by organic anion transporting proteins (OATPs) and H^+-monocarboxylic acid cotransporters—albeit not by design—where it is responsible for most of its dramatic cholesterol-lowering activity. This is nicely illustrated

by Maede *et al*. [21] who administered a microdose of atorvastatin (about 20 mg) to healthy volunteers with or without coadministration of rifampicin (600 mg; an OATP inhibitor) or itraconazole (200 mg; a CYP3A4 inhibitor). The PK plasma area under the curve of atorvastatin increased 12-fold with rifampicin co-administration and stayed the same with itraconazole. In addition, two of atorvastatin's metabolites, 2-hydroxy-atorvastatin and 4-hydroxy-atorvastatin, are active as well. Thus, there is a systemic PK/PD disconnect due to fortuitous hepatic uptake, and neither the relatively low systemic exposure nor the high MW is a liability. Indeed, Lennernäs pointed out that "*The effect of statins is not closely related to the plasma concentration of the drug and its active metabolites. Instead, several reports have shown that the pharmacological response was better correlated with the daily dose of statins*" [20].

3.3.3 Candesartan Ciletexil

Candesartan ciletexil breaks the "rule of five" on several fronts: MW, log *P*, and HBAs. However, candesartan ciletexil is a prodrug by design, and it is completely and rapidly converted to candesartan in the gastrointestinal tract during absorption. Candesartan itself—an angiotensin II receptor antagonist for the treatment of hypertension—is a polar dianionic compound (it contains tetrazole and carboxylic acid functional groups) that would not be absorbed appreciably if it had not been for generation of a prodrug.

3.3.4 Montelukast

Montelukast breaks both the MW and the log *P* rule. The latter is not surprising because the endogenous substrates of the target, the leukotriene D4 (cysLT1) receptor, are very lipophilic as well. However, montelukast has sub-nanomolar potency and this effectively counters, among others, the high plasma protein binding driven by its lipophilicity and being a carboxylic acid.

3.3.5 Orlistat

Orlistat is a reversible covalent lipase inhibitor for weight loss and has a log *P* of 8.6. Not surprisingly, orlistat is practically insoluble in water and the bioavailability is $<1\%$. However, the target is in the intestine and systemic exposure is not required for efficacy.

3.3.6 Ritonavir

Ritonavir is a HIV protease inhibitor and breaks the MW rule by a considerable margin with a MW of 720.9 Da. The log *P*, 4.9, is relatively high as well. Overall, ritonavir has quite undesirable properties and the development was encumbered with significant physical form and formulation issues [22]. Originally, ritonavir was marketed as an oral liquid and a semi-solid capsule. Both formulations contained ethanol to aid solubility because ritonavir is not bioavailable from the solid state. However, after a few years, problems arose because a different conformational polymorph appeared in the production process with even lower solubility and, therefore, greatly reduced bioavailability. This threatened supplies and necessitated immediate reformulation. To solve these issues, the original capsules were replaced with refrigerated gelcaps. Clearly,

the physicochemical properties were a serious liability, but ritonavir was a life-saving treatment for AIDS and the benefits outweighed its distinct disadvantages.

3.3.7 Telmisartan

Telmisartan—another angiotensin II receptor antagonist for the treatment of hypertension—is part of a large group of compounds, which breaks the "rule of five" based on its log P value, but the log D value is much lower. The log P value of telmisartan—a carboxylic acid—is 7.3, but the log D value at pH = 7.4 is between 3 and 4. The solubility at neutral pH is relatively low, but the permeability is acceptable and the absolute bioavailability in humans is 43%, which is higher than most other angiotensin II receptor antagonists.

3.3.8 Intrinsic and Extrinsic Attenuating Circumstances

An appropriate term—also used in social sciences—to describe these compounds (3.3.1–3.3.7) is "positive deviants." This analysis is not meant to be complete, but it does highlight a few valuable points. Many positive deviant drugs that break the "rule of five" are associated with specific attenuating circumstances.

Intrinsic attenuating circumstances:

- The MW rule may be broken because of the presence of a large number of halogen atoms (e.g., amiodarone and levothyroxine). Some argue that the molecular volume is a better measure for absorption than MW and halogen atoms have a disproportionately high atomic weight compared with their volume. This, has resulted in the proposal to calculate the "corrected" atomic weight with fluorine, chlorine, bromine, and iodine counting for 5.2, 19.2, 26.3, and 37.4 Da, respectively, instead of 19.0, 35.5, 79.9, and 126.9 Da, respectively [23].
- Some drugs with a high MW are prodrugs that are cleaved presytemically (e.g., candesartan ciletexil and valganciclovir). Note that several drugs on the market are prodrugs, but not by design (e.g., sibutramine and terfenadine).
- Several acidic and basic drugs break the "rule of five" based on their log P value, but the more physiologically relevant log D value at the intestinal pH is much lower and compatible with absorption (e.g., gefitinib and telmisartan). Unfortunately, calculation of the log D at a specific pH requires calculation of the pK_a, which can be problematic.
- Some drugs with multiple HBDs and HBAs form internal hydrogen bonds, which reduces the number of HBDs and HBAs able to interact with water. See the following sections for more details.

Extrinsic Attenuating Circumstances:

- Some drugs act locally in the gastrointestinal tracts (e.g., miconazole and orlistat).
- Some of drugs that break the "rule of five" display a significant systemic PK/PD mismatch (e.g., alendronic acid and atorvastatin) and the low systemic exposure is not a liability. This determination requires an in-depth knowledge of the biological mechanism and site of action.

- Many drugs that violate the "rule of five" are for life-threatening diseases and the pharmacological benefits outweigh the disadvantages (e.g., ritonavir and saquinavir).

Setting aside the positive deviant drugs described above, the number of compounds that break the "rule of five" without attenuating circumstances and are commercially successful is quite limited and they usually distinguish themselves by being extremely potent (e.g., montelukast). An analysis of drugs that have been launched between 2006 and 2008 comes up with similar conclusions [24]. For example, lapatinib has an MW of 581.1 Da and a *c*log *D* of 6.0. Not surprisingly, the absorption of lapatinib is incomplete and variable and the human daily dose is 1.25 g. However, lapatinib targets a life-threatening disease: breast cancer and other solid tumors. Another example is aliskiren, which is a direct renin inhibitor for primary hypertension. The MW of the free base is 551.8 Da and it has six HBDs and a PSA of 157 $Å^2$. Although the solubility of aliskiren is very good, the absolute bioavailability is only 2.6% and the absorbed fraction is estimated to be 5% [25]. In addition, the interindividual variability in human aliskiren PK is large and there is a large, negative food effect [26]; apple juice and orange juice impact the exposure negatively as well [27]. The human PK of aliskiren are governed by a combination of (i) limited passive permeability, (ii) active intestinal efflux mediated by P-glycoprotein (P-gp) and uptake by OATPs, and (iii) a significant hepatic first-pass effect mediated by hepatic uptake and active biliary secretion of the parent compound. However, aliskiren can be detected in the kidney—the site of action—weeks after administration, even though the drug is undetectable in the plasma [24]. Fortuitously, aliskiren accumulates in renin granules, which explains the long-lasting renin angiotensin blockade beyond the half-life of aliskiren [28]. Thus, alsikiren is another classical example of a drug with poor PK driven by specific "rule of five" violations, but a PK/PD disconnect renders the systemic exposure less relevant. These examples and several others led Teague to conclude,

> "*Where there is a choice in a therapeutic area between a lipophilic agent that violates the rules and one with a better balance of properties, senior management would still be best advised to back the latter. Imperfect agents might simply validate a target and encourage competitors to enter with a drug having the improved properties* [24]."

In summary, although differences in physicochemical properties exist across targets (and companies), the majority of existing drugs for most target types follow the "rule of five." Thus, the conclusion is that extreme physicochemical properties are more likely to be accompanied by poor pharmaceutical and PK properties and, to be a successful drug, other advantageous properties—such as potency, a long off-rate from the receptor, or active distribution to the site of action—have to make up for those deficits and/or the drug should target a life-threatening disease with few therapeutic alternatives. However, as research progresses and more drugs reach the markets, the bar will be raised for drugs targeting very serious disorders as well. A good example is telaprevir (MW is 679.9 Da and the log *P* is 4.0), a very promising drug for the treatment of hepatitis C virus (HCV) infection [29]. The bioavailability in preclinical and clinical studies with the initial form was poor (2% or less) and variable. Indeed, scientists from Vertex commented that marble was more soluble than telaprevir. Extensive formulation development was required to come up with a stable amorphous dispersion

with good bioavailability, 20–40%. Despite these heroic and laudable efforts, the dose and the daily pill burden are still remarkably high: 750 mg three times a day. It is likely that in this highly competitive therapeutic area [30], new drugs will appear on the market with a superior regimen and lower cost of goods. Indeed, Merck focused on improving the PK properties and hence the dose in their second generation HCV NS3 protease inhibitor, narlaprevir, which is currently in clinical trials [31]. In conclusion, the nature of the target and its tissue distribution, the therapeutic area, and the competitive landscape—and therefore the target candidate profile—should define *up front* the acceptable physicochemical space and whether potential liabilities associated with breaking the "rule of five" are acceptable or not.

One class of drugs that deserve special recognition is natural products [32]. Despite pronounced "rule of five" violations, many natural products and in particular macrocyclic natural products such as cyclosporine, erythromycin, and tacrolimus display acceptable PK properties, including bioavailability. Several reasons have been proposed for this such as involvement of uptake transporters and extensive internal hydrogen bond formation and reduced conformational flexibility [33]. A recent nuclear magnetic resonance (NMR) and gas phase computational analysis [34] has shown that cyclosporine forms four internal hydrogen bonds, which reduces the number of HBDs from 5 to 1. The internal hydrogen bonds also reduce the effective PSA (not predicted by the TPSA) and lead to a more compact conformation with a lower molecular volume. These factors could explain the remarkably good oral bioavailability of cyclosporine in humans, 30% [34]. Unfortunately, natural product research has been scaled back by most pharmaceutical companies.

Overall, these data reemphasize the importance of physicochemical parameters. However, physicochemical parameters not only influence absorption, they also affect other ADME parameters such as clearance and distribution and the appropriate physicochemical "sweet spot" should be defined for each parameter and each project. This dependence will be illustrated in Section 3.10 after a brief outline of ADME assays deployed in drug discovery to guide design. In addition, in Section 3.10, the dependence of ADME properties on physicochemical parameters will be explored in an *integrated* and *prospective* manner, whereas the relationships highlighted in this section relied mostly on a *retrospective* analysis.

3.4 *IN VITRO* ADME STUDIES

With advances in bioanalysis and the availability of both specific cell lines and subcellular fractions, it has become possible to incorporate a range of *in vitro* ADME end points more effectively in drug discovery with the hope of steering projects towards superior ADME properties. Of course, as will be illustrated below, the ADME properties have to be weighed against other attributes such as potency and selectivity. This has resulted in complex screening cascades incorporating end points that reflect potency, efficacy, selectivity, toxicity, and ADME properties. The following *in vitro* ADME end points are routinely assessed in drug discovery:

- Permeability in Caco-2 or Madin–Darby canine kidney (MDCK) cells or parallel artificial membrane permeability assay (PAMPA)

- Efflux in MDCK or Lilly Laboratories cell-porcine kidney (LLC-PK) cells over-expressing transporters such as P-gp and breast cancer resistance protein (BCRP)
- Metabolic stability in microsomes, S9, cytosol, hepatocytes, or recombinant CYP enzymes
- Reversible CYP inhibition
- Mechanism-based or time-dependent CYP inhibition
- CYP induction
- Plasma protein binding or drug binding in other matrices such as microsomes or brain
- Blood to plasma partitioning

Each of these *in vitro* ADME assays should be incorporated in the overall screening cascade for discovery projects, although the order and extent may vary from project to project. An example of an effective screening cascade for a CNS target is presented in Fig. 3.3.

3.4.1 Permeability

Absorption across the epithelial cell layer in the gastrointestinal tract is the first hurdle a drug has to overcome to reach a systemic site of action. Caco-2 cells, a well-differentiated intestinal cell line derived from human colorectal carcinoma, display many of the morphological and functional properties of the intestinal epithelial cell layer and, consequently, have been used extensively as an *in vitro* model to study absorption. Indeed, a correlation between flux across Caco-2 cells and the fraction absorbed has been established. However, growing these cells is time consuming. Therefore, other cell lines that take less time to grow to confluence, such as MDCK and LLC-PK cells, have been introduced. In these experiments, the bidirectional flux, from apical to basolateral and from basolateral to apical, is determined. If there is asymmetry, this suggests the involvement of uptake or efflux transporters (see below). Non cell-based assays, such as the PAMPA, are used as well. PAMPA employs a barrier made of phospholipids (e.g., phosphatidyl choline and egg lecithin) solubilized in a long-chain hydrocarbon. Despite the experimental convenience of PAMPA, most companies prefer using cell-based assays, because they are a more realistic reflection of the *in vivo* absorption barrier.

3.4.2 Metabolic Stability

Most of the *in vivo* clearance of drugs is driven by metabolism and the most important drug-metabolizing enzymes belong to the cytochrome P450 (CYP) family. The liver is the most prominent site of metabolism and, therefore, hepatocytes and/or hepatic subcellular fractions are used to study metabolism. CYP enzymes and flavin-containing monooxygenases (FMOs) are present on the cytoplasmic side of the endoplasmatic reticulum and as such are active in liver microsomes. Liver microsomes from various species are ubiquitously available and are used in a higher throughput manner to determine the propensity of drugs to be metabolized by CYP enzymes and FMOs. Other enzymes contribute to metabolism as well, but a different subcellular fraction

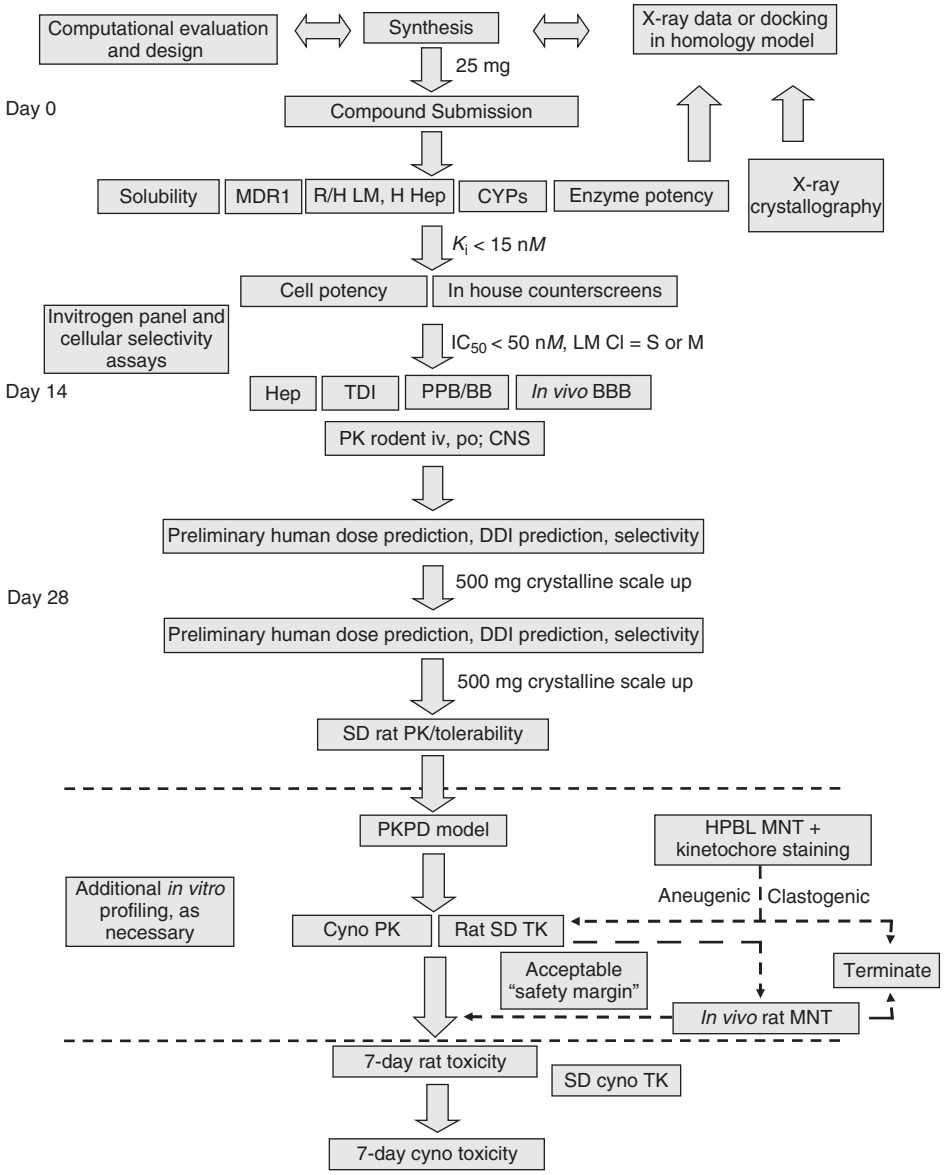


Figure 3.3 Screening cascade for a CNS target.

may be required. For example, aldehyde oxidases, alcohol dehydrogenases, and aldehyde dehydrogenases are present in cytosol. To get the drug exposed to the full hepatic complement of drug-metabolizing enzymes, including phase 2 enzymes such as uridine diphosphate glucuronosyltransferases (UGTs) and sulfotransferases (SULTs), hepatocytes can be used and the availability of cryopreserved hepatocytes has advanced the field considerably. Thus, it is important to look at different matrices to make sure that specific metabolic pathways are not missed. In the metabolic stability experiments, the compounds are incubated for a short period (20–60 min for microsomes and 1–4 h with

hepatocytes) with appropriate cofactors and the abundance of the parent compound is monitored as a function of the incubation time. Sometimes, the percentage parent compound remaining is reported, but the data are usually converted to a half-life, $t_{1/2}$, and CL_{int} or CL_{hep} values using appropriate scaling factors.

In most cases, microsomes are used as the tier 1 assay with hepatocytes as a downstream, lower capacity tier 2 assay. However, as projects progress and more is known about the metabolic fate of compounds, the cascade can and should be revised accordingly. For example, if direct glucuronidation appears to be the major metabolic pathway, hepatocytes should be used as the tier 1 assay. Metabolism is not limited to the liver and can occur in other organs, such as the intestine, kidneys, and lungs, as well. Micosomes of those tissues are commercially available. Usually, most attention is paid to metabolism in the liver, but if it becomes clear that hepatic metabolism cannot explain the observed *in vivo* clearance, other tissue should be considered and microsomes of those tissues can be incorporated in screening cascades.

3.4.3 Phenotyping

To better understand the human PK and to assess the likelihood of a compound being the "victim" of a drug–drug interaction, it is important to identify the individual enzymes responsible for the metabolism of a compound. This is commonly done for CYP and/or UGT enzymes. The experiments involve incubating the compound with recombinant enzymes or incubating the compound with microsomes and using specific antibodies or chemical inhibitors.

3.4.4 Drug–Drug Interaction

Several drugs, such as cerivastatin, cisapride, terfenadine, and mibefradil, have been withdrawn because of severe drug–drug interaction, and therefore, this should be addressed early on in the screening cascade. The most ubiquitous drug–drug interactions are the result of inhibition of CYP enzymes. Reversible CYP inhibition can be determined by looking at the inhibition of metabolism of probe substrates to specific metabolites mediated exclusively by a specific CYP enzyme. Examples of commonly used probe substrates are provided in Table 3.4.

If throughput is an issue, the percent inhibition of turnover of the probe substrates at a specific concentration of the drug candidates can be determined. Alternatively, multiple concentrations of the drug candidates can be used and an IC_{50} value can be determined. The IC_{50} value provides value information to assess the risk of a clinical drug–drug interaction, but quantitative predictions also require information about the anticipated exposure of the "perpetrator" and the fraction of the "victim" drug being metabolized by the inhibited CYP.

Mechanism-based CYP inhibition should be explored as well, because it inactivates the enzyme either in a covalent manner (heme or apoprotein adduct formation) or via metabolite–intermediate complex formation and requires *de novo* synthesis of the enzyme. Indeed, many of the most pronounced drug–drug interactions are due to mechanism-based CYP inhibition [35] and it is remarkably prevalent these days; an analysis by Zimmerlin *et al*. [36] indicated that 4% of 400 marketed drugs were positive, whereas 23% of 4000 randomly selected new chemical entities from Novartis

TABLE 3.4 Probe Substrates and LC-MS/MS Detection Method Used for Inhibition of Specific Cytochrome P450 Enzymes

Enzyme	Reaction	Ionization Mode	MRM Transition
CYP1A2	Phenacetin-*O*-deethylation	+	152 → 110
CYP2B6	Bupropion hydroxylation	+	256 → 238, 139
CYP2C8	Paclitaxel-6α-hydroxylation	+	870 → 286, 105
CYP2C9	*S*-Warfarin-7′-hydroxylation	+	325 → 179
CYP2C9	*S*-Warfarin-7′-hydroxylation	—	323 → 177
CYP2C9	Tolbutamide-4′-hydroxylation	+	287 → 171, 89
CYP2C9	Tolbutamide-4′-hydroxylation	—	285 → 186
CYP2C19	*S*-Mephenytoin-4′-hydroxylation	+	235 → 150
CYP2C19	*S*-Mephenytoin-4′-hydroxylation	—	233 → 190
CYP2D6	Dextrometorphan-*O*-demethylation	+	258 → 199, 157
CYP2E1	Chlorzoxazone-6-hydroxylation	+	186 → 130
CYP2E1	Chlorzoxazone-6-hydroxylation	—	184 → 120
CYP3A4/5	Midazolam-1′-hydroxylation	+	342 → 324, 297, 203
CYP3A4/5	Testosterone-6β-hydroxylation	+	305 → 269

drug discovery projects were positive. Mechanism-based inhibition is characterized by the following:

- NADPH-dependent inactivation;
- concentration-dependent inactivation;
- time-dependent inactivation;
- enzyme activity not restored by dialysis;
- inactivation rate diminished in the presence of competing substrate;
- spectral shift from 450 nm.

The most common assay format involves preincubation of the drug candidate in microsomes followed by dilution and incubation with an appropriate probe substrate (Table 3.4). The preincubation may give rise to a marked decrease in activity of the CYP enzyme and, hence, reduced turnover of the probe substrate specific for that enzyme. Because the experiment is carried out in a time-dependent manner, this phenomenon is usually called *time-dependent inhibition* (TDI), but it is important to realize that the observed inhibition can be due to both mechanism-based inhibition and the formation of metabolites that are potent CYP inhibitors. To increase throughput, the experiment can be performed with a single preincubation period (usually 30 min) with multiple concentrations of the investigative drug in the absence and presence of NADPH. The extent of TDI is reflected by the shift in the IC_{50} value, hence the name "IC_{50} shift assay" [37]. To enable clinical prediction, multiple concentrations and multiple preincubation times are desirable, and K_I and k_{inact} values can be obtained after mathematical transformation of the data. As explained for reversible CYP inhibition, quantitative predictions also require information about the anticipated exposure of the "perpetrator" and the fraction of the "victim" drug being metabolized by the inhibited CYP.

Both reversible and mechanism-based CYP inhibition have been performed in hepatocytes, but the use of microsomes is most ubiquitous.

3.4.5 CYP Induction

CYP induction assays are usually not tier 1 assays in drug discovery projects because the prevalence of induction is limited. However, for certain classes of drugs, induction is quite common. The gold standard assay is incubation of the drug candidates with multiple batches of fresh hepatocytes for 24–48 h. After removal of the supernatant and washing of the cells, the hepatocytes are incubated with specific probe substrates (Table 3.4) to determine via functional activity if the expression of specific CYP enzymes is up-regulated. Alternatively, mRNA expression can be monitored. Cell viability should be monitored as well, because it could circumvent detection of CYP induction. This is clearly a low throughput assay and for projects with specific induction issues pregnane X receptor (PXR) binding is usually employed to enable sufficient throughput and an adequate turnaround time.

3.4.6 Plasma Protein Binding and Tissue Binding

Knowing the non-covalent binding of a drug to plasma proteins or lipids in tissue is important because the free concentration is the presumed driver of efficacy (the "free drug hypothesis" or "free drug concept") [38,39]. However, it is not a parameter that should be "optimized" (see below). In plasma, most drugs bind extensively to plasma proteins such as albumin (neutral compounds and acids) and α-acid glycoprotein (basic compounds). In tissues, binding is much less specific and mainly reflects affinity for interaction with phospholipids. Several assays are available to determine the free or unbound drug concentration. Ultrafiltration and ultracentrifugation are used, but the gold standard is considered to be dialysis. Dialysis can be performed with individual tubes or using a 96-well block with buffer on one side and plasma or tissue homogenate on the other side. After incubation, both sides are sampled. If binding is high (plasma protein binding in excess of 99% is not uncommon for higher MW and/or lipophilic drugs), a very sensitive LC-MS/MS assay has to be used. For proper PK/PD modeling, it is important to know the free tissue concentration at the site of action and this can be obtained by multiplying the total concentration and the free fraction in the relevant tissue. In the absence of the involvement of uptake or efflux transporters, the free drug concentration in plasma and tissue should be the same, although the free fraction in plasma and tissue, and hence the total drug concentration in plasma and tissue, may be quite different.

3.4.7 Drug Transport Assays

Studying drug transport has become more common because many drugs are to some extent substrates or inhibitors of uptake or efflux transporters [40]. The most extensively studied drug transporter is P-gp (also called *MDR1*) because it can (i) limit intestinal absorption, (ii) enhance biliary excretion, and (iii) limit brain and/or tumor penetration. Thus, for central targets requiring sufficient brain exposure, it is essential to incorporate a P-gp assay early on in the screening cascade. Efflux in Caco-2 or MDCK cells may be indicative of the compound being a P-gp substrate, but the evidence is not conclusive until confirmed by efflux reduction with a specific P-gp inhibitor. A more common assay involves cells overexpressing P-gp. Cell lines for other transporters such as BCRP, OATs, OCTs, OATPs, and MRPs are available as well, but are usually not used

extensively in drug discovery, because they do not seem to play a major role in drug disposition with the exception of specific classes of drugs (such as hepatic uptake of statins via OATPs).

Many pharmaceutical companies have higher throughput ADME screening facilities for these assays (3.4.1–3.4.7). The assays are heavily automated and use 96- or 384-well format [41,42]. The end points usually involve sample analysis via LC-MS/MS. A capacity of 200–1500 compounds per week through the tier 1 assays (usually metabolic stability in microsomes and reversible CYP inhibition) is not uncommon [43]. Although these data can and should be used to influence design in drug discovery, they are not rigorous enough for regulatory submissions and more customized assays are required for that purpose.

3.5 *IN VIVO* PHARMACOKINETIC STUDIES

In vivo PK studies continue to play an important role in drug discovery and development. The purpose of these studies is threefold:

- determination of exposure in preclinical species to inform subsequent PD or efficacy studies
- determination of exposure in preclinical species to aid in the interpretation of toxicology studies
- determination of PK parameters in preclinical species to aid in the identification of compounds with appropriate human PK.

The availability of sensitive and selective mass spectrometers interfaced with LC systems has greatly facilitated the incorporation of *in vivo* PK studies in drug discovery. In most cases, exposure in plasma is measured, but other matrices such as blood, urine, feces, or specific tissues of pharmacological or toxicological interest may be sampled as well. Some companies rely heavily on *in vivo* PK data in their screening cascades necessitating higher throughput approaches. The most common approaches are the following.

3.5.1 Cassette or "n-in-one" Dosing

A mixture of drugs is administered to the same animal resulting in a significant reduction in the number of animals used for PK studies. With the advent of highly selective and sensitive triple quadrupole mass spectrometers operating in the multiple reaction monitoring (MRM) mode (Fig. 3.4), it is feasible to determine the exposures of all drugs in the cassette [44,45]. Nevertheless, it is important to select the drug candidates in the cassette such that common metabolic pathways do not interfere with the detection of the parent compounds. For example, drug candidates that differ in MW by 16 or 32 Da should be avoided. Drug–drug interaction via inhibition of CYPs or other metabolic enzymes is not inconceivable either [46]. However, this would generally result in a higher exposure and these "false positive" compounds would be captured in subsequent experiments. Thus, the risk of "false negatives" (i.e., compounds with lower exposure in a cassette than as a single agent) is low. Nevertheless, the dose is usually lowered in cassette studies to reduce the risk of drug–drug interaction. In

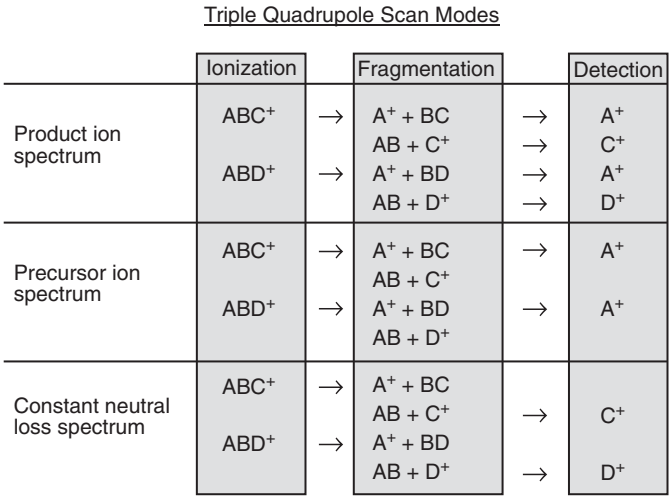


Figure 3.4 Principles of the product ion, precursor ion, and constant neutral scan modes feasible with a triple quadrupole mass spectrometer.

addition, a reference compound with well-established PK and a close analog of the drug candidates under investigation is usually included in every cassette. Generally, the substantial increase in output outweighs the (manageable) risks.

3.5.2 Sample Pooling

To avoid the complications associated with cassette dosing as described above, some companies employ sample pooling. In this approach, individual compounds are dosed and plasma samples are subsequently pooled and analyzed with a triple quadrupole mass spectrometer (e.g., cassette-accelerated rapid rat screen [47]). This technique eliminates the risk of PK drug–drug interaction, but the risk of bioanalytical interference due to metabolites remains.

In addition, equipment such as automated blood sampling, multiplexing, and column switching with staggered parallel analysis is routinely used to enable higher throughput for *in vivo* PK studies. Although MRM on a triple quadrupole mass spectrometer is the mainstay in bioanalysis, there is a recent push to use high resolution MS [48]. The data are gathered with a single stage of mass analysis, which would result in a loss of selectivity, but the high resolution makes up for that. Moreover, it is now possible to monitor both the parent compound and metabolites without prior knowledge about the nature of the metabolites.

3.6 TISSUE DISTRIBUTION

Knowledge of tissue distribution can be valuable to relate to efficacy or toxicity in a particular tissue. Some compounds are taken up in tissues and are present in much higher concentrations than in plasma either due to uptake transporters, preferential binding to cellular components (e.g., lipids or melanin), or pH-mediated trapping of the charged form of the drug (e.g., lysosomal trapping). Alternatively, there can be

active efflux of drug candidates out of tissues such as the brain. The latter can be either desirable (e.g., limited brain penetration of cytotoxic oncology drugs that target non-CNS tumors) or undesirable (e.g., limited brain penetration for CNS agents). A comprehensive view of tissue distribution can be obtained by quantitative whole body autoradiography (QWBA), but this requires radiolabeled material, which is usually not available in drug discovery. Moreover, QWBA cannot distinguish the parent compound from metabolites. A more convenient, but time-consuming, approach is excision of individual tissues, followed by homogenization and quantitation by LC-MS/MS. This technique does not require radiolabeled material and is very sensitive; the MRM mode prevents significant interference by endogenous components. However, each tissue must be examined separately and the distribution within each tissue is lost.

Imaging via matrix-assisted laser desorption/ionization (MALDI) was introduced in the early twenty-first century and it has been successfully used to detect drugs and their metabolites in tissues [49]. Tissue or whole body slices are obtained in the same manner as for QWBA, but the slices are subsequently coated with a finely dispersed matrix solution (such as sinapinic acid in methanol). The solvent solution extracts the analytes out of the tissue and the matrix itself allows ionization after irradiation with a laser. In most cases, detection in the MS/MS mode is preferred to prevent interference by the abundant matrix ions. The tissue distribution of either the parent drug and/or its metabolites is obtained by scanning the laser across the tissue with a spatial resolution of about 50 μm. Although significant progress has been made, this technique still suffers from limited sensitivity and lengthy data acquisition. The latter can be addressed by acquiring data in the MS mode with a high resolution mass spectrometer and extracting the images for the parent drug and its metabolites post-acquisition [50].

3.7 METABOLITE IDENTIFICATION

LC-MS/MS is a very powerful tool for metabolite identification. Metabolites generated from *in vitro* incubations or *in vivo* studies can be detected with relative ease and this information can be communicated to medicinal chemists to address specific metabolic liabilities in the next generation of compounds. Several mass spectrometric scan modes are available to aid in the assignment of structures of metabolites (Fig. 3.4). The most common scan modes are as follows:

- *Product Ion Scan.* A protonated or deprotonated metabolite is selected with the first mass analyzer and subsequently fragmented by collision with an inert gas. Finally, the fragments are analyzed according to their mass or, to be precise, their mass-to-charge (m/z) ratio in the second mass analyzer.
- *Constant Neutral Loss Scan.* In this scan mode, the first and second mass analyzers are scanned with a fixed offset that corresponds to a common fragment lost from the parent drug and/or its metabolites in the collision cell. For example, glucuronide metabolites can be detected by performing a 176-Da constant neutral loss scan in the positive ion mode and glutathione adducts can be detected with a 129-Da constant neutral loss scan.
- *Precursor Ion Scan.* A precursor ion scan allows monitoring of ions (corresponding with the parent drug and/or its metabolites) that generate a common fragment ion. In this scan mode, the first mass analyzer is scanned and the second mass

analyzer is held constant at the mass-to-charge ratio of the common fragment ion. For example, glutathione adducts can be detected by a m/z 272 precursor ion scan in the negative ion mode [51].

Note that not all scan modes are available on all types of mass spectrometers due to design limitations. However, the most common type of mass spectrometer, the triple quadrupole instrument, allows all three of these scan modes. With the availability of high resolution time-of-flight, orbitrap, and Fourier-transform mass spectrometers, it is possible to obtain accurate mass values in either the MS or the MS/MS modes, which greatly facilities interpretation of spectra and assignment of metabolite structures. In the MS mode, an accurate mass provides—with a reasonable degree of confidence—the molecular formula of the metabolite and thereby the type of biotransformation involved. In the MS/MS mode, accurate mass values of the fragment ions facilitate interpretation of the MS/MS spectrum and, consequently, assignment of the structure of the metabolite. An additional feature of a high resolution mass spectrometer is the ability to apply a mass defect filter to the data file to remove endogenous matrix-related ions [52,53]. The mass defect refers to the fractional mass of the parent compound and its metabolites obtained using the accurate mass for each atom. For example, the exact mass of atorvastatin is 558.2521 Da and the corresponding mass defect is 0.2521 Da. Metabolites with minimal structural changes relative to the parent compound (i.e., +O, +2O, and $-CH_2$) can be detected with a mass defect filter similar to that used for the parent compound because the change in fractional mass is minimal (−0.0051, −0.0102, and −0.0156 Da for +O, +2O, and $-CH_2$, respectively). For conjugates such as sulfates or glucuronides, the mass defect filter should reflect the change in the fractional mass of the conjugates.

Despite these advances in the field of MS, it is still very hard to identify the exact site of metabolism, in particular for the most common metabolic process: aliphatic and aromatic hydroxylation. Identification is usually limited to determination of the moiety of the molecule that was metabolically transformed and a Markush structure of the metabolite is obtained. Sometimes, the exact site of metabolism may be unambiguous (e.g., for simple N-dealkylations), but for common pathways, such as oxidation, it is frequently required to isolate the metabolite and obtain NMR data to identify the exact structure. Additional tools available to facilitate metabolite identification are as follows:

- isotope patterns of $[M + H]^+$ or $[M - H]^-$ ions; metabolite identification is greatly facilitated by the presence of atoms with a unique isotope pattern such as chlorine and bromine;
- presence of a radiolabel (usually ^3H or ^{14}C);
- chemical derivatization;
- hydrogen–deuterium exchange [54];
- studies with close in analogs.

More recently, it has been advocated to integrate quantitative bioanalysis and metabolite identification in one experiment. This can most easily be achieved using a high resolution instrument operating in the MS mode [48]. The high resolution ensures appropriate selectivity for detection and quantitation of the parent compound.

In addition, the data can be interrogated for the presence of metabolites and the accurate mass measurement can provide the molecular formula of the metabolite. A subsequent experiment may be required to obtain structural information via product ion scans. Alternatively, the instrument can automatically switch from MS to MS/MS mode (provided it has this capability) in a data-dependent manner. It remains to be seen whether the quality of the quantitative information is sacrificed.

Another area of metabolite identification that is receiving more and more attention is the detection of adducts that are indicative of bioactivation [55,56]. Many drugs are bioactivated and there is some (mostly circumstantial) evidence that reactive intermediates may give rise to (idiosyncratic) toxicity. Although the reactive intermediates cannot be detected, it is possible to trap them with agents such as glutathione (abundant in the liver and an effective nucleophile by virtue of its cysteine sulfhydryl group), potassium cyanide (a "hard" nucleophile for detection of iminium species), and methoxylamine and semicarbazide (to detect aldehydes). LC-MS/MS assays have been implemented to facilitate detection of these adducts. For example, glutathione adducts can be detected by a 129-Da neutral loss scan in the positive ion mode or a m/z 272 precursor ion scan in the negative ion mode [51]. Some labs have taken this a step further and are examining covalent binding to cellular components (usually proteins). Nakayama *et al*. [57] looked at the correlation between covalent binding and glutathione adduct formation and TDI using a data set of 42 structurally diverse compounds. First, they found no statistically significant correlation between the formation rate of glutathione adduct formation and the extent of covalent binding; several compounds, including pioglitazone and ritonavir, showed extensive covalent binding ($>$50 pmol/min/mg protein as defined by Evans *et al*. [55]). It would be interesting to see whether a correlation would have appeared if other trapping agents, such as cyanide and methoxylamine, would have been incorporated in the assay. Second, there was no statistically significant relationship between the enzyme inactivation rate (as assessed by the TDI assay) and the extent of covalent binding. Third, the authors calculated the intrinsic clearance for forming reactive metabolites by summing (i) the intrinsic clearance for forming reactive metabolites based on the formation rate of glutathione adducts and (ii) the intrinsic clearance for forming reactive metabolites based on the enzyme inactivation rate in the TDI assay. Fortunately, there was a statistically significant correlation between the intrinsic clearance for forming reactive metabolites as defined above and the extent of covalent binding with no obvious false negatives (clean in the TDI assay and/or glutathione trapping assay, but more than 50 pmol/min/mg protein in the covalent binding assay). Thus, this suggests that the combination of the TDI and the glutahione trapping assays can serve as a surrogate to assess the risk for covalent binding. Many scientists have advocated the use of covalent binding as a surrogate for toxicity, but it has been shown that there is no clear relationship between the extent of covalent binding and hepatotoxicity [58]. Moreover, it requires radiolabeled material and, therefore, is not very practical in drug discovery. A most interesting finding in the analysis of Nakayama *et al*. [57] is that atorvastatin was determined to have covalent binding of 352 pmol/min/mg protein, which far exceeds the guideline of 50 pmole/min/mg protein [55]. Nevertheless, atorvastatin is remarkably safe and commercially successful and the low dose, 10–80 mg, may be the main reason for this. It has been well established that the vast majority of drugs withdrawn from the market due to idiosyncratic adverse drug reactions (IADRs) or having a black box warning due to IADRs have a dose of $>$100 mg [59].

In conclusion, the degree of concern about formation of reactive metabolites should be guided by the following parameters:

- clinical dose and exposure;
- duration of therapy (short vs long duration);
- therapeutic area (life threatening or not?);
- target population (young vs old, healthy vs diseased);
- first in disease/class or not;
- lead compound versus backup;
- species differences (bioactivation may be unique to one species).

3.8 *IN SILICO* ADME MODELING

A substantial benefit from higher throughput *in vitro* ADME screening is that there is now a wealth of data available that can be used to build sophisticated *in silico* ADME models. *In silico* models have been available for years and some of them are commercially available. However, most of the commercially available models are of limited value because the chemical diversity of the data used to build the model is limited in size and, sometimes, the data were obtained from the literature using different experimental protocols. High throughput ADME screening—generating in excess of 10,000 data points per year—has overcome these hurdles and, consequently, models have been built for most *in vitro* ADME endpoints: permeability, metabolic stability in microsomes and hepatocytes, microsomal binding, plasma protein binding, reversible and time-dependent CYP inhibition, and so on. [60]. Although global models are useful, in some cases, it may be better to build a local model that better defines the chemical space of interest. The models involve a range of chemical descriptors and/or the structures of the compounds in the training set. Different statistical methods (regression methods: partial least squares; Bayesian methods; supervised learning methods: decision trees, support vector machine; and neural networks) can be used as well. The output of the model can either be categorical or numerical. Besides the output, the confidence in the prediction is a critical parameter. The confidence in the prediction is usually derived from (i) the structural similarity of the compound under investigation with those in the training set and (ii) the number of structurally similar compounds (nearest neighbors). This, combined with an objective validation of the model using compounds not in the training set, guides the ultimate use and incorporation of the model in drug discovery. For example, Lee *et al*. [61] built an *in silico* model for metabolic stability in microsomes and showed that 72% of the compounds without close nearest neighbors were predicted correctly, whereas 88% of the compounds with more than 33 nearest neighbors were predicted correctly. The same phenomenon is also apparent from the data in Fig. 3.5 showing the correlation between the *in silico* and *in vitro* human liver microsomal extraction ratio. The color of the symbols characterizes the similarity with the compounds in the training set and the shape of the symbols reflects the number of nearest neighbors in the training set. Generally, the dark gray squares in Fig. 3.5 (similarity score ≤ 0.7; number of nearest neighbors ≤ 7.8) reflect compounds with less similarity and fewer nearest neighbors and, not surprisingly, the predictions are less

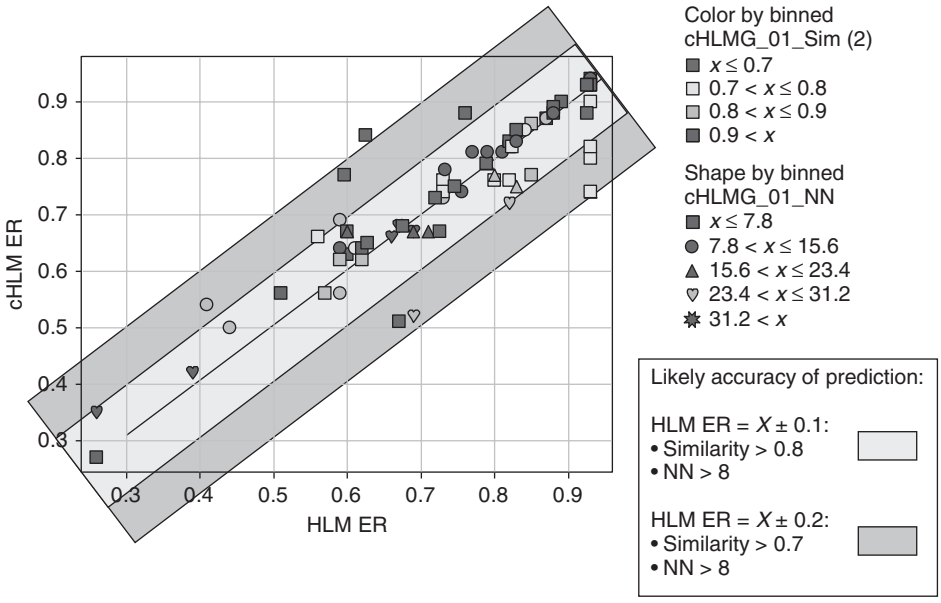


Figure 3.5 Extraction ratio (ER) measured in human liver microsomes (HLM) versus the calculated ER in HLMs derived from an *in silico* metabolic stability model. The color of the symbol is indicative of the similarity (Sim) and the shape is indicative of the number of nearest neighbors (NN). (See color insert.)

accurate. It is important that the data set to build the *in silico* models is regularly updated to reflect the evolving chemical space.

Although models serve a very useful purpose, most models are generally not meant to replace experimental data—or at least not at this stage. The main goal should be to increase the *probability* of identifying compounds with optimum properties, such as metabolic stability or permeability, and to identify the SAR of the ADME properties. Indeed, the models can be used to triage compounds *before* synthesis and speed up the lead optimization process. In-house data at Genentech show that the number of compounds with good microsomal stability (CL_{hep} in human liver microsomes <6.2 mL/min/kg) increased from $<10\%$ of all new compounds synthesized to slightly more than 20% after implementation of an *in silico* metabolic stability model in 2010, but without dramatic changes in the portfolio. Of course, there are many reasons to synthesize particular compounds and if there is a good design hypothesis (e.g., based on specific interactions with the target derived from the crystal structure or a homology model), synthesis should proceed despite unfavorable ADME predictions. Ideally, multiple *in silico* models, including potency models, are available and used in a parallel manner to triage very large virtual libraries. Unfortunately, the progress with building reliable *in silico* models for potency appears to lag behind the advances with *in silico* ADME models. The *in silico* ADME model can also be used to triage compounds already synthesized before submission to *in vitro* ADME assays if the capacity of the assays is limited. For example, if the model is highly confident that a particular compound is metabolically unstable, there is no reason to submit it for experimental verification.

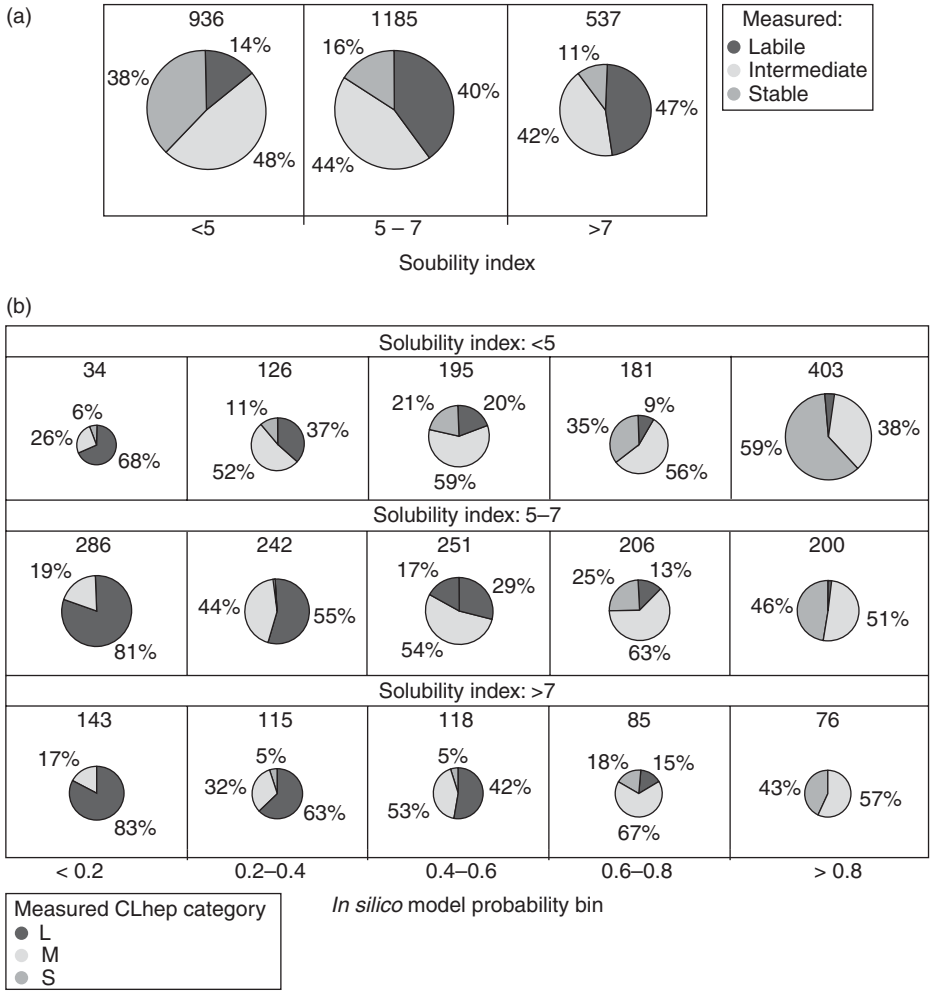


Figure 3.6 (a) Relationship between solubility index (sum of the log *P* and the number of aromatic rings) and the metabolic stability in human liver microsomes. The following definitions for metabolic stability are used: stable $CL_{hep} < 6.2$ mL/min/kg (gray), intermediate 6.2 mL/min/kg $< CL_{hep} < 15$ mL/min/kg (light gray), and unstable $CL_{hep} < 15$ mL/min/kg (dark gray). (b) Relationship between the predicted probability of being stable in the human liver microsomes metabolic stability *in silico* model (0, infinitely small probability of being stable; 1, highest probability of being stable) and the measured metabolic stability as a function of the solubility index. (None of the compounds examined here were in the training set of the model.) The definitions are the same as in (a). (See color insert.)

The value of *in silico* models is also illustrated by the data in Fig. 3.6. Figure 3.6 shows the predicted and experimental human liver microsomal metabolic stability data of more than 2600 compounds not in the training set as a function of the solubility index. The *in silico* metabolic stability model is built with a training set of *in vitro* data from close to 20,000 diverse compounds obtained under consistent experimental conditions. The solubility index is the sum of the log *P* and the number of aromatic

rings and the combination of these two parameters is a reasonably reliable indicator of the experimental solubility and can be routinely calculated [62,63]. A solubility index of 7 or more indicates a high *likelihood* of very low solubility, $\leq 1\ \mu M$, and a solubility index of 5 or less is usually desirable. First, the experimental data in Fig. 3.6a show that compounds with a solubility index of <5 have a 3.5-fold greater probability of being *in vitro* metabolically stable than compounds with a solubility index of 7 or more (38% vs 11%). The latter probably reflect the increased likelihood of large lipophilic compounds with many aromatic rings to be both poorly soluble and extensively metabolized. Second, Fig. 3.6b shows that the ability of the *in silico* metabolic stability model to identify compounds that are experimentally stable in human liver microsomes is better for compounds with a low solubility index. For compounds that have a predicted probability of being stable >0.8 (0 = infinitely small probability of being stable; 1 = highest probability of being stable) close to 60% are indeed metabolically stable if the solubility index is <5, whereas about 40% are experimentally stable if the solubility index is more than 7. Thus, both the solubility index and the *in silico* metabolic stability model can serve as a tier 0 screen to improve the *probability* of identifying compounds with better PK and pharmaceutical properties.

The output of an *in silico* CYP3A4 time-dependent CYP inhibition model is presented in Fig. 3.7. The TDI model is based on a training set of close to 2000 compounds with midazolam or testosterone as the probe substrates of 3A4 and using an "IC_{50}

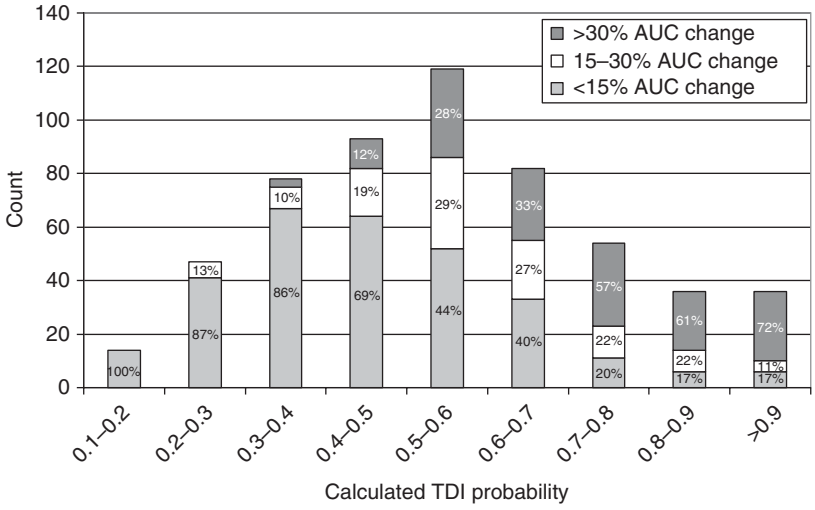


Figure 3.7 Relationship between the predicted probability of displaying time-dependent inhibition (TDI) of CYP3A4 using an in-house *in silico* model (0, very low probability for TDI; 1, high probability for TDI) and the measured extent of TDI using an "IC_{50} shift" protocol with the fold-change in area under the percent activity remaining versus inhibitor concentration curve (i.e., AUC change) rather than the traditional fold-change in IC_{50} value as the experimental endpoint to determine the magnitude of TDI [64]. (None of the compounds examined here were in the training set of the model.) The following definitions for TDI are used: low risk < 15% AUC change (light gray), intermediate risk between 15% and 30% AUC change (white), and high risk > 30% AUC change (dark gray).

shift" protocol with the fold-change in area under the percent activity remaining versus inhibitor concentration curve rather than the traditional fold-change in IC_{50} value as the experimental endpoint to determine the magnitude of TDI [64]. The output of the model is the probability of showing TDI with 1 representing a very high probability for TDI and 0 a very low probability. Although using hard "cutoff" values is not advisable, a probability of 0.7 or more indicates a high probability of displaying TDI, whereas below 0.4 the risk is much lower. Nevertheless, there is a finite probability for both "false negatives" and "false positives"—as is the case for any *in silico* model despite an effort to minimize them while building the model. "False negatives" are of limited concerns because compound synthesis will proceed and the experimental data will ultimately supersede the *in silico* data. "False positives" may greatly concern project teams because those compounds may be dropped from further pursuit. Although that may be alarming, other compounds that are presumably predicted to display superior properties are synthesized instead. Thus, the detrimental impact may be minimal and the overall positive impact of incorporating *in silico* models far outweighs this risk.

As mentioned before, some models are commercially available and models such as MetaSite and StarDrop have proven to be relatively reliable. Although the underlying computational algorithms are different for MetaSite and StarDrop, both are capable of predicting the site of metabolism considering both the intrinsic reactivity of each potential site to oxidative metabolism by CYP enzymes as well as the accessibility of the site of metabolism to the active oxy-heme species in the CYP enzymes. Using the literature and in-house compounds, it was shown that both MetaSite and StarDrop are capable of predicting the right site of metabolism about 60–90% of the time for CYP2C9, CYP2D6, and CYP3A4 [65]. Note that both software packages predict the site of metabolism, but not the exact metabolic pathway nor the rate of metabolism. An example of the use of MetaSite is presented in Fig. 3.8 (only partial structures are shown because the structures are still proprietary). A potent compound was identified, but its clearance in mice was very high, 157 mL/min/kg. MetaSite predicted the site of metabolism as the 4-position of the piperidine ring. Next, a compound was synthesized with a substituent at the 4-position and the clearance was much lower, 8.6 mL/min/kg, and the free fraction in plasma was slightly increased as well. Subsequent metabolite identification efforts with the first compound indicated that the piperidine ring was indeed the site of metabolism. Unfortunately, the potency of the second compounds was more than 1000-fold lower.

There is another inherent and implied advantage of using *in silico* ADME models and that is that it enables evaluation of hits from the HTS screen. In the past, the

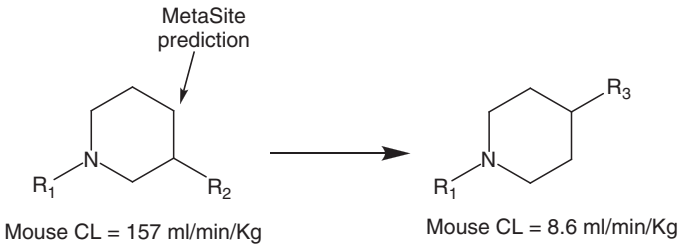


Figure 3.8 MetaSite prediction of the site of metabolism for a high clearance compound inspiring chemical modification that resulted in a low clearance compound.

decision to pursue a certain chemical series was based on its potency and chemical intuition. However, it has been shown that different medicinal chemists view attractiveness of a compound or series remarkably different [66]. The *in silico* ADME models allow early incorporate of appropriate ADME parameters and have them weighted as important as endpoints such as potency and selectivity. This may be particularly important because on many occasions, it is easier to optimize potency and selectivity than ADME properties. Thus, early and appropriate incorporation of *in silico* ADME models followed closely by experimental verification will facilitate identification of drugable chemical space and subsequently speed up lead optimization.

3.9 MULTIPARAMETER OPTIMIZATION

Currently, the lead optimization process is much more complex than it was even 10 years ago because the sheer quantity of parameters and data has grown almost exponentially. The ability of a human, even an experienced drug "hunter," to optimize more than three parameters in a simultaneous manner is severely limited. Thus, computational tools are available that allow the user to incorporate multiple parameters and rank order the compounds. A simple model resembling a traffic light that can be used to assess absorption in the gastrointestinal tract is presented in Table 3.5 [23] and incorporates solubility, lipophilicity, the corrected MW (see above), PSA, and the number of rotatable bonds. The "cutoff" values for each property are presented in Table 3.5 and the overall score is a summation of those for the individual parameters with a score of 0 being ideal and 12 being highly undesirable. An analysis showed that 70% of the oral drugs have an overall score of 2 or less, whereas HTS hits from 20 screens showed a Gaussian-like distribution peaking at a score of 5. Additional parameters can be incorporated easily and these parameters can be either computational or experimental in origin.

Another example is the model used to assess whether a compound is likely to be able to cross the blood–brain barrier [67]. The parameters included are *c*log *P*, *c*log *D*, MW, TPSA, number of HBDs, and pK_a. Each parameter has a desirable and less desirable range and a transition resulting in a score of 0–1 with 1 being most desirable. Subsequently, the individual scores for the six parameters are summed with 6 being the highest possible score. Most CNS drugs have a score of 4 or more. Not surprisingly, experimental verification indicates that a high score is accompanied by a high passive permeability (P_{app} value in MDCK cells), low P-gp liability (efflux ratio

TABLE 3.5 Traffic Light Multiparameter Optimization Tool

Traffic Light Color	Traffic Light Value	Solubility (mg/L)	*c*log *P*	Corrected Molecular Weight	PSA (Å^2)	Rotatable Bonds
Green	0	≥50	≤3	≤400	≤120	≤7
Yellow	1	10–50	3–5	400–500	120–140	8–10
Red	2	<10	>5	>500	>140	≥ 11

Source: Data from Lobell *et al*. [23].

in P-gp overexpressing MDCK cells), and low unbound intrinsic clearance ($CL_{int,u}$ in human liver microsomes).

The two examples described above were focused on physicochemical parameters and each input parameter had the same weight. It also possible to include a range of experimental data and more sophisticated tools allow the user to adjust the weight of each parameter and to incorporate experimental uncertainty [68].

Human dose predictions in early drug discovery (in contrast to dose predictions performed during candidate nomination, which are based on more sophisticated human PK prediction and PK/PD analysis) is another form of multiparameter optimization, although it may not be perceived by many as such. For example, the dose can be predicted with Equation 3.1 using the following parameters:

$C_{ss,\,avg}$, cellular potency IC_{50} value;

CL, human clearance derived from human liver microsomes or hepatocytes or rat–human scaling;

τ, dosing interval (usually 12 or 24 h);

F, back-calculated bioavailability assuming that the clearance is solely hepatic (1 - CL/Q) or based on preclinical bioavailability data.

$$\text{Dose} = \frac{C_{ss,avg} \cdot CL \cdot \tau}{F} \tag{3.1}$$

It is important to emphasize that this is not meant to be an accurate dose prediction. It is merely meant to provide an indication about the order of magnitude and it can be used to categorize compounds/chemical series.

A more comprehensive overview of multiparameter optimization in drug discovery is presented in Ref. 69.

3.10 INCORPORATION OF ADME DATA IN DRUG DISCOVERY TO IMPROVE PHARMACOKINETICS

Incorporation of all relevant parameters in drug discovery in a *prospective* effort to find a drug with the "perfect" balance of properties is extremely challenging and time consuming and frequently it is necessary to compromise. Indeed, "*... the level of in vitro potency of a lead for its intended target(s) is the key parameter at the beginning of a drug discovery program. This idea is extended further in lead generation-optimization based on the assumption that the most potent molecules in vitro are generally more likely to translate into more effective therapies, possessing a greater overall safety profile and requiring lower therapeutic doses. However, with regard to the first assumption, from this study and many others in the literature, it seems that the safety profile (ADMET and off-target related effects) is more likely to be inversely correlated with the level of target potency given their opposing relationship with regard to key physicochemical properties*" [9]. The same authors showed that *in vitro* potency can only explain roughly 30% of the variation in dose. Having the right screening cascade to balance out the various properties is critical and prevents late stage surprises. A somewhat generic example for a CNS target is presented in Fig. 3.3.

Table 3.6 summarizes the key physicochemical parameters—both molecular and specific structural features—that influence various ADME properties listed in the third column. Note that many physicochemical parameters are not independent of each other. For example, if the MW increases, the number of rotatable bonds usually increases as well, and the PSA and the total number of HBDs and HBAs are also correlated. The interconnectivity between the physicochemical parameters and the ADME properties have been illustrated with several literature and in-house examples.

The first PK hurdle that oral drugs encounter is absorption. Absorption is influenced by both solubility and permeability. In general, solubility increases with increasing HBD and HBA count, PSA, and the fraction of the bonds that can freely rotate, but is negatively influenced by increasing log P, log D, and the number of aromatic rings [63,70]. However, the exact opposite is usually true for permeability and this necessitate identification of a physicochemical "sweet spot" for absorption. The situation is even more complex if absorption is limited by intestinal efflux. A nice example illustrating the level of complexity was presented by Lévesque *et al*. [71]. The authors were trying to identify a rennin inhibitor for blood pressure control. Earlier, lipophilic compounds with a basic amine functionality were potent and orally bioavailable but suffered from CYP 3A4 and hERG inhibition. To address the latter liabilities, while maintaining potency, the size and lipophilicity of the leads were reduced significantly, but this resulted in negligible bioavailability. Increasing the dose improved the bioavailability somewhat, but the clearance did not change. This, combined with the observation that the majority of the drug was detected in the feces after oral dosing in bile duct cannulated rats, suggested P-gp limited absorption. Indeed, P-gp KO (knockout) mice and rats codosed with elacridar (a P-gp inhibitor) showed improved bioavailability (but no change in clearance). All these compounds showed very poor permeability in LLC-PK cells and, therefore, it was impossible to determine the efflux ratio in LLC-PK cells overexpressing P-gp. Thus, the authors prepared more lipophilic compounds (log $D_{7.4} > 1.7$) and this increased the passive permeability significantly ($P_{app} > 10 \times 10^{-6}$ cm/s). The latter did not eliminate the efflux as detected in the P-gp overexpressing

TABLE 3.6 Molecular and Structural Features that Influence ADME Properties

Molecular Features	Structural Features	ADME Properties
Molecular weight	Hydrogen bond donors	Solubility and dissolution rate
Molecular volume	Hydrogen bond acceptors	Permeability
pK_a	(Aromatic) rings	Absorption
log P	% sp^3 carbon atoms	Plasma protein binding and tissue binding
log $D_{7.4}$	Rotatable bonds	Red blood cell partitioning
Polar surface area		Clearance (metabolic, transporter-mediated, passive renal, or biliary)
% Polar surface area		Distribution (including transporter-mediated uptake or efflux)
		Inhibition and induction of drug-metabolizing enzymes and transporters

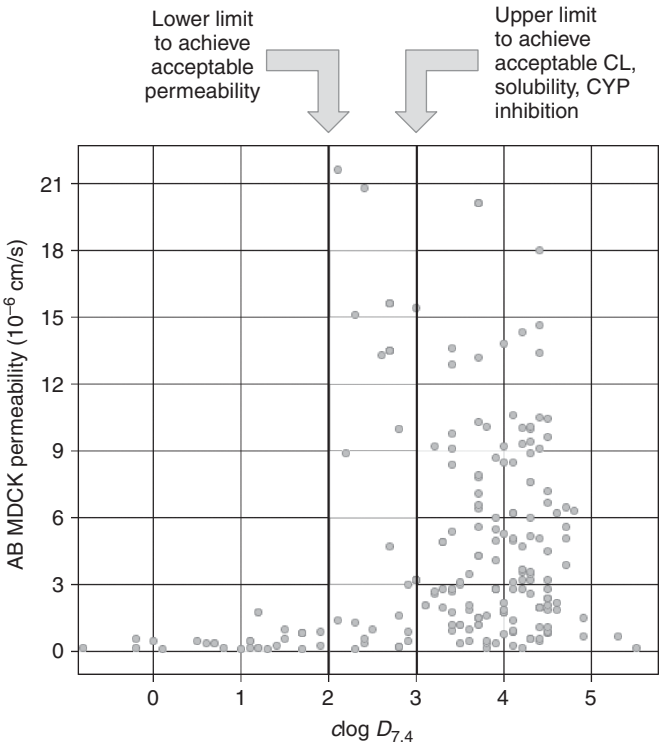


Figure 3.9 Relationship between *c*log $D_{7.4}$ and the measured A to B permeability in MDCK cells for a large number of compounds synthesized for an in-house project.

LLC-PK cells (efflux ratio $\approx$ 20), but the *in vivo* impact was drastically reduced and the bioavailability of many of the more lipophilic compounds exceeded 25%. However, not all the more lipophilic compounds showed good bioavailability and this was ascribed to a significant first-pass effect. Thus, the lipophilicity was modulated to balance potency, bioavailability, CYP inhibition, and hERG. The same phenomena are illustrated in Fig. 3.9 based on data from an in-house project. The AB permeability in MDCK cells is plotted as a function of the *c*log $D_{7.4}$ and illustrates that a *c*log $D_{7.4}$ of at least 2 is needed to achieve acceptable permeability, although it does not guarantee it. Data not presented here indicated that a *c*log $D_{7.4} > 3$ increased the probability of poor solubility and high clearance, resulting in a narrow "sweet spot."

There appears to be a Gaussian or parabolic relationship between the log $D_{7.4}$ and the extent of absorption and bioavailability [72]. At log $D_{7.4} < 0$, a compound's solubility is usually good, but its permeability across membranes is poor resulting in limited absorption. In contrast, at log $D_{7.4} > 5$, the membrane permeability is adequate, but the solubility is low, which significantly reduces absorption. In addition, metabolic clearance in the intestine and liver, which affects F_g and F_h, tends to be higher for compounds with log $D_{7.4} > 5$. However, this effect appears to be modulated by MW with the log D range that leads to good absorption and metabolic stability being wider at low MW than at high MW [73]. The PSA is another important parameter for passive membrane permeability with a PSA in excess of 120 Å^2 associated with poor absorption

[74] and in excess of 90 Å^2 associated with poor brain penetration [17]. This effect can be ascribed to decreased membrane permeability for polar compounds. In contrast, a very low PSA (<50 Å^2) can lead to increased intestinal and hepatic extraction [72].

Two common approaches to identify absorption issues are the maximum absorbable dose (MAD) and the dose number (D_0).

$$\mathrm{MAD} = k_a \cdot S \cdot V_{\mathrm{intestine}} \cdot T \tag{3.2}$$

where k_a is the absorption rate constant (usually obtained from PK studies in preclinical species); S the solubility of the drug in a relevant medium; $V_{\mathrm{intestine}}$ the volume of water in the small intestine available to dissolve the drug (usually 250 mL in humans); and T the small intestine transit time (usually 4 h for the small intestine in humans).

$$D_0 = \frac{D}{(V_{\mathrm{intestine}} \cdot S)} \tag{3.3}$$

where D is the dose, $V_{\mathrm{intestine}}$ the volume of water in the small intestine available to dissolve the drug (usually 250 mL in humans), and S the solubility of the drug in a relevant medium.

The parameters influencing the MAD and D_0 are fraught with experimental uncertainty, most of all the solubility. The solubility determined in discovery is usually kinetic in nature and the true thermodynamic solubility is not available until later. Moreover, the solubility is strongly influenced by the solvent system (buffered solvents or fed or fasted state simulated intestinal fluid) and its pH and the physical form of the drug (amorphous or crystalline, the most stable polymorph or not, presence of excipients, etc.). In addition to the extent of solubility, the dissolution rate should be taken into consideration as well and both the MAD and D_0 ignore this aspect. Nevertheless, the MAD is easy to calculate and provides a crude guide; if the MAD is <10% of the anticipated human dose, one should consider the possibility of dose-limited absorption. Similarly, if the dose number is >10, absorption could be incomplete. Note that these numbers are merely indicators of absorption risk and should be used as impetus to explore absorption further.

Once absorbed, the clearance is usually the most important parameter. An analysis of the routes of elimination of the 200 most prescribed drugs indicated that for about 72% of the drugs, metabolism was the major route of drug elimination, whereas renal elimination and biliary clearance were prevalent for 25% and 3%, respectively, of the top 200 drugs [75]. For those drugs predominantly cleared via metabolism, the CYP enzymes were the major contributors to metabolism. Considering the trend toward higher MW compounds (as illustrated in Table 3.2), it is reasonable to assume that passive renal clearance will be low for most drugs and that the major component of clearance will be either metabolism or active excretion into the bile, urine, or intestine. It is well established that increasing the lipophilicity can lead to increased clearance. In addition, increasing the PSA and the HBDs and HBAs decreases the clearance [72]. However, it is imperative to focus on the unbound or free clearance. The unbound clearance can be obtained as follows:

$$\mathrm{CL_u} = \frac{\mathrm{CL}}{f_u} \tag{3.4}$$

where CL is the total clearance, CL_u the unbound or free clearance, and f_u the unbound fraction in plasma.

In keeping with the concept that in most cases, efficacy is driven by the amount of unbound drug available, it is imperative to convert the relevant PK parameters to their unbound equivalent (e.g., AUC_u and CL_u). Too often, drug discovery efforts lead to an increase in lipophilicity and, consequently, a smaller f_u, while maintaining or sometimes even improving CL. This could result in a false sense of success. Instead, teams should focus on the CL_u value (which reflects the free amount of drug to drive efficacy), which may have increased dramatically. It is also important not to optimize f_u or use it to prioritize compounds for further studies. There is no optimum f_u and, indeed, there are many successful drugs with small free fractions [38]. Interestingly, there is a difference between the effect of plasma protein binding *in vitro* and *in vivo* [39]. *In vitro* an increase in f_u from 0.1 to 0.5 by changing the structure of the compound increases the unbound concentration five-fold as the total concentration remains the same. *In vivo* after an oral dose, an increase in f_u from 0.1 to 0.5 will increase the unbound concentration only if the intrinsic hepatic clearance decreases at the same time. If the intrinsic hepatic clearance stays the same, an increase in f_u from 0.1 to 0.5 will not increase the unbound concentration and will reduce the total concentration. Thus, the goal should be to optimize the physicochemical parameters such that it leads to a lower intrinsic clearance. Obach *et al*. [76] performed a detailed analysis of 670 drugs administered to human intravenously, which allowed determination of the clearance and volume of distribution. Their analysis showed that the unbound clearance increases dramatically with the lipophilicity with hydrophilic compounds with a log P between 0 and 1 having a median unbound clearance of 8.5 mL/min/kg and very lipophilic compounds with a log $P > 4$ having a median unbound clearance of 150 mL/min/kg. The relationship between *c*log P and the free fraction for three chemical series in one project is illustrated in Fig. 3.10 and shows that the free fraction steadily decreases with increasing lipophilicity, although the three chemical series are offset from each other. Another in-house analysis involving more than 2200 compounds across all projects came to the same conclusion. Specifically, 54% of the compounds with a measured log $D_{7.4} > 4$ have a free fraction smaller than 0.01, whereas only 6% of the compounds with a measured log $D_{7.4}$ between 1 and 2 have a free fraction smaller than 0.01. Conversely, 66% of the compounds with a measured log $D_{7.4}$ between 1 and 2 have a free fraction >0.1, whereas only 3% of the compounds with a measured log $D_{7.4} > 4$ have a free fraction >0.1. A graph derived from the data compiled by Obach *et al*. [76] is presented in Fig. 3.11 and displays the correlation between f_u and CL_u. The implication of this graph is that lowering the lipophilicity may actually have a dual effect by lowering CL_u *and* increasing the free fraction. Of course, lowering the lipophilicity may reduce the potency (because of less interaction with the target) and decrease the permeability. An analysis (Fig. 3.12) based on rat PK data for more than 50 compounds in one project shows how sensitive the relationship can be. If the *c*log P value is <1, more than 90% of the compounds have an unbound clearance of <170 mL/min/kg, whereas with a *c*log P value >2, only 32% of the compounds have an unbound clearance of <170 mL/min/kg.

A nice example of optimization of physicochemical parameters is illustrated by the discovery of crizotinib. Initially, crizotinib was positioned as a c-MET inhibitor. Later it was also found to inhibit anaplastic lymphoma kinase (ALK) and it was launched as treatment for non-small-cell lung cancer in 2011. The original lead candidate, PHA-665752 (Fig. 3.13), demonstrated poor PK and pharmaceutical properties,

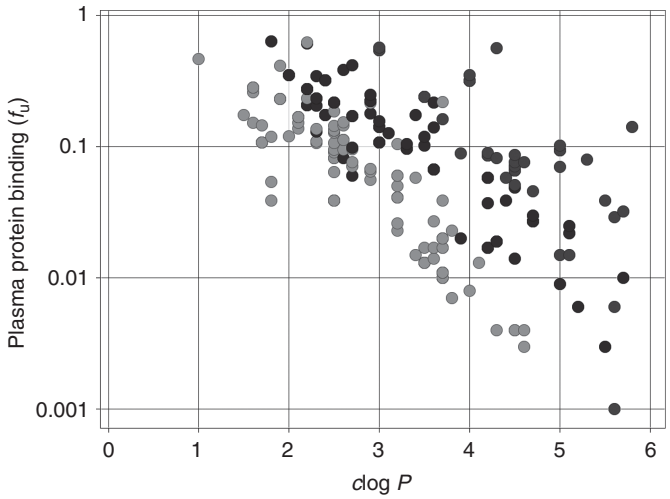


Figure 3.10 Relationship between *c*log *P* and the measured free fraction (f_u) in plasma for a large number of compounds synthesized for an in-house project.

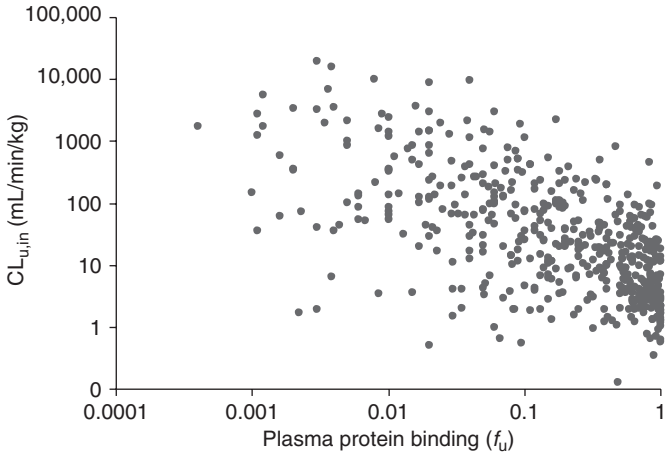


Figure 3.11 Relationship between the measured free fraction (f_u) in plasma and the unbound intrinsic clearance ($CL_{u,in}$) derived from data presented in Ref. 76.

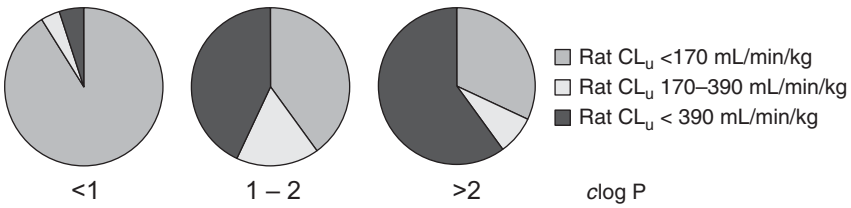


Figure 3.12 Relationship between *c*log *P* and unbound clearance (CL_u) in rats for more than 50 compounds synthesized for an in-house project.

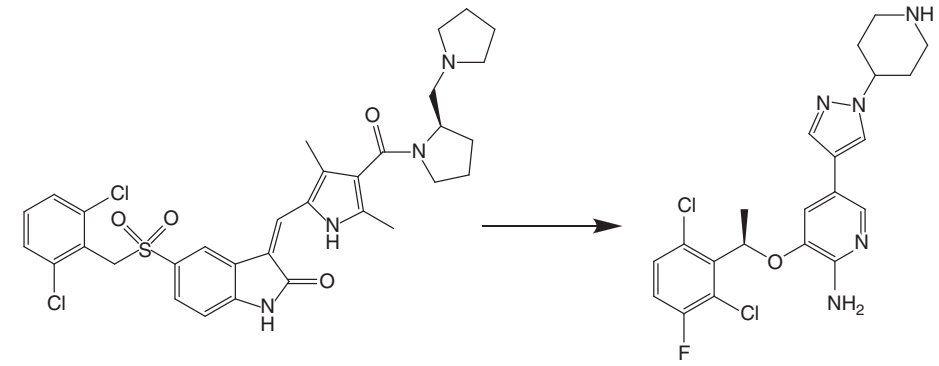
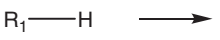
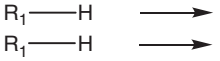
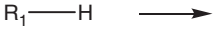
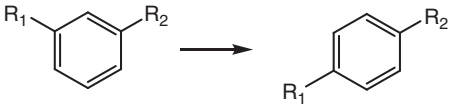
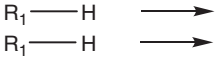


Figure 3.13 Optimization of the original lead c-MET inhibitor, PHA-665752, leading to the dual c-MET/ALK inhibitor, crizotinib.

which limited its development as a clinical candidate despite good cell potency (c-MET cell $IC_{50} = 9nM$) and selectivity [77]. For example, the clearance of PHA-665752 in rats was higher than hepatic blood flow, 77 mL/min/kg [78], and it had low permeability and poor solubility at pH 7.4, 0.9 μg/mL, which prevented nomination as a potential intravenous treatment [77]. PHA-665752 having a high MW, 641.6 Da, and being quite lipophilic ($\log D_{7.4} = 3.2$ and $\text{LipE} = \text{pIC}_{50} - \log D_{7.4} = 4.8$), the goal was to identify equally potent compounds with improved physicochemical properties. Detailed SAR studies, fueled to a large extent by cocrystal structures of compound bound to c-MET, led to PF02341066, crizotinib. Crizotinib still makes the same key interaction with the c-MET protein, but is much smaller with a MW of 450.3 Da. Considering the lower lipophilicity, $c\log D_{7.4} = 2.0$, and a similar cellular potency in the c-MET assay, 8 n*M*, the LipE of crizotinib is improved to 6.1 [77]. Moreover, the solubility is improved and the same applies to the preclinical PK (e.g., the clearance values in rat and dog are 29 and 9 mL/min/kg, respectively, and the bioavailability values are 63% and 65%, respectively). The human dose is 250 mg twice daily (BID), the half-life is 43–51 h, and there is no food effect, which defines an attractive clinical profile [79].

An interesting computational approach to improve clearance was proposed by Keefer *et al*. [80]. They performed a very comprehensive pairwise analysis of a large database of compounds with metabolic stability data in human liver microsomes, permeability data in RRCK cells (MDCK cells with lower levels of endogenous transporters), and P-gp-mediated efflux in MDCK cells overexpressing P-gp. They determined the 20 most frequently occurring transformations in their database and their mean change and standard deviation associated with the three *in vitro* ADME parameters described above. A few examples and their impact on microsomal metabolic stability and RRCK permeability are presented in Table 3.7. They also showed that transformations that involve multiple steps are frequently additive. However, their analysis also showed that similar transformations in very different molecules may not always have the same net effect and sometimes they even have the opposite effect. Nevertheless, it provides a

TABLE 3.7 Statistical Changes in Metabolic Stability in Human Liver Microsomes and RRCK Permeability Associated with the Most Common Transformations Based on a Pairwise Analysis of a Very Large Database

Transformation	HLM Stability		RRCK Permeability	
	Mean Change	%Increase/% Same/% Decrease	Mean Change	%Increase/% Same/% Decrease
	21.9	45/41/12	−0.2	24/47/28
	2.7	24/56/18	−0.5	17/55/26
	10	38//42/19	−3.1	16/35/48
	20.5	45/45/9	−1.0	20/48/31
	−15.4	12/49/37	−0.1	10/60/19
	−19.9	10/43/45	1.1	33/47/18
	−32.0	11/33/54	−3.1	23/28/47

Listed are the mean change of the property for each transformation and the percentage of the pairs with the property increasing, staying the same, or decreasing.
Source: Data are from Ref. 80.

simple (computational) rule of thumb to increase the likelihood to identify compounds with improved ADME properties.

Finally, it is important to recognize that multiple enzymes can contribute to the metabolism of compounds and, if the choice of *in vitro* studies and preclinical PK studies is not comprehensive enough, the data can be deceiving. A classical example is carbazeran (Fig. 3.14). Carbazeran is relatively stable in microsomes across species and the PK in dogs looks good with a CL of 11.5 mL/min/kg and a bioavailability of 68% [81]. In sharp contrast, the clearance in humans was 38 mL/min/kg, which is higher than hepatic blood flow, and carbazeran was not detected in the plasma after an oral dose of 350 mg. Subsequently, it was determined that human metabolism was mainly via aldehyde oxidase leading to oxidation of the phthalazine moiety. Moreover, dogs have very low levels of aldehyde oxidase, which explains the good PK in dogs. Had the investigators used either hepatocytes, S9 or cytosol, to examine the metabolic stability of carbazeran across species before nominating the compound for development, they would have identified the aldehyde oxidase liability in humans. Remarkably, compounds failed in the clinic as recent as 2011 due to extensive aldehyde oxidase metabolism in humans (e.g., FK3453 [82] and BIBX1382 [83]; see Fig. 3.14 for structures) despite the ability to identify the liability preclinically by doing the right *in vitro* studies or performing a monkey PK study.

Another important parameter to consider is the volume of distribution and, not surprisingly, Obach *et al*. [76] have shown that the volume of distribution, and in particular the unbound volume of distribution, increases with the lipophilicity. In addition, the volume of distribution decreases with the PSA and the number of HBDs and HBAs. The combined data for clearance and volume of distribution are summarized in Table 3.8 by charge state. Note that the V_d and CL are generally much lower for acids

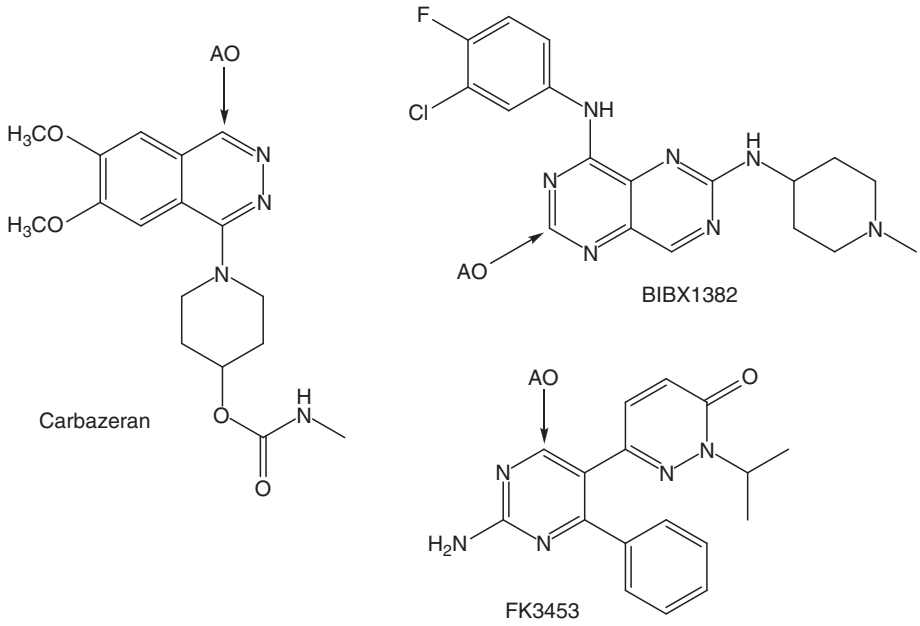


Figure 3.14 Structures of the aldehyde oxidase (AO) substrates carbazaran, FK3453, and BIBX1382. The sites of AO metabolism are indicated by arrows.

TABLE 3.8 Comparison of Median Values for Total and Unbound V_d and CL Values Relative to Charge Type

	Median V_d	Median $V_{d,u}$	Median CL	Median CL_u
Acids ($n = 159$)	0.22	2.2	2.1	17
Neutrals ($n = 173$)	1.1	5.5	4.0	21
Bases ($n = 267$)	2.3	9.2	7.6	23
Zwitterions ($n = 68$)	0.83	2.0	2.4	3.8

Source: Data from Obach *et al*. [76].

than bases, but the differences are reduced after correcting for plasma protein binding. If the drug target is in the brain, particular attention should be paid to CNS distribution. As described above, a TPSA in excess of 90 $Å^2$ is associated with poor brain penetration [17]. This is partly due to poor passive permeability, and it also appears that the probability for P-gp-mediated efflux increases with a TPSA above 70–80 $Å^2$, as illustrated in Fig. 3.15 for data from multiple chemical series for one particular in-house CNS target.

Obviously, there are numerous other parameters that play a role in drug design and ADME is only one of them. For example, the desire to increase potency frequently results in larger and more lipophilic compounds. This necessitates finding a "sweet spot" that represents the best overall balance. A generalization of the dependence of various ADME parameters on the key physiochemical properties is summarized in Table 3.9.

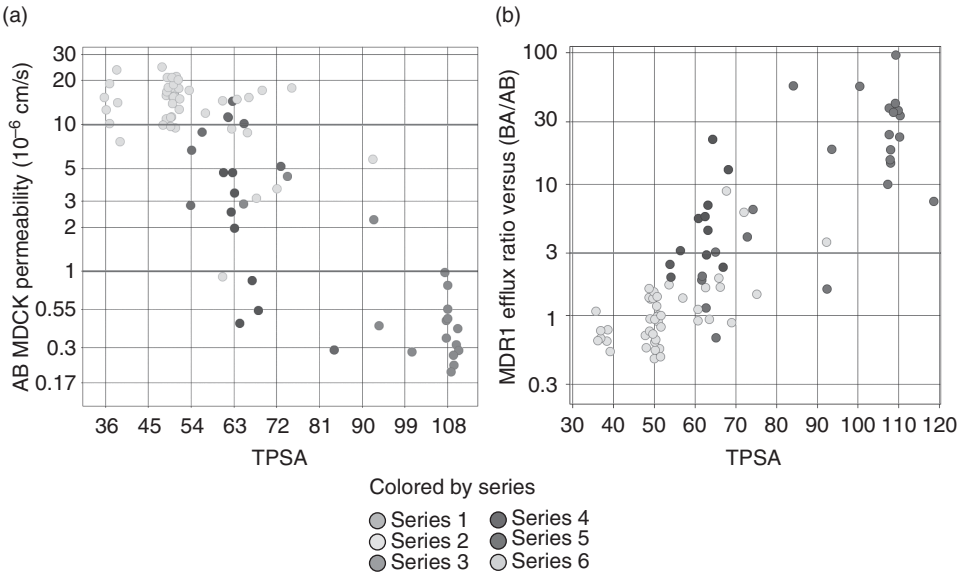


Figure 3.15 Relationship between TPSA and the A to B permeability in MDCK cells (A) and the B to A/A to B efflux ratio in MDCK cells overexpressing MDR1 for a large number of compounds synthesized for an in-house project. (a) Permeability versus TPSA and (b) MDR1 efflux ratio versus TPSA.

TABLE 3.9 Generalization of the Dependence of Various ADME Parameters on Physicochemical Properties

	log D	MW	PSA	HBA + HBD
Solubility	↓	↓	↑	↑
Permeability	↑	↓	↓	↓
F_a	↑	↓	↓	↓
F_h	↓	=	↑	↑
CL	↑	—	↓	↓
F	Gaussian (↑ then ↓)	↓	Gaussian (↑ then ↓)	↓
V_d	↑	—	↓	↓
Plasma protein binding	↑	—	—	

[a]The arrows indicate an increase, no change, or decrease in the pharmacokinetic parameter in one of the rows as a function of an increase in the physiochemical parameter in one of the columns.

Finally, ADME scientists play a critical role in other aspects of drug discovery such as identification of drug–drug interaction liabilities, determination of exposure in PD and efficacy studies, PKPD analysis of preclinical data, and human PK and dose predictions. Detailed descriptions of these aspects are beyond the scope of this chapter and the readers are referred to others chapters in the Encyclopedia of Drug Metabolism for these components.

3.11 CONCLUSIONS

It is realistic to state that incorporation of ADME parameters early on drug discovery has had and will continue to have a positive impact on drug development. However, drug candidates have become larger and more lipophilic to meet potency criteria and this has in general a negative impact on ADME parameters and drug development in general. Before engaging in an extensive drug discovery effort, it is important to clearly define the acceptable target candidate profile and identify any attenuating circumstances that could influence the profile. Next, a thorough effort to establish the SAR of all key parameters (potency, selectivity, permeability, clearance, etc.) should be conducted, and finally, the physicochemical "sweet spot" that provides the best balance of properties should be identified.

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