7 Drug Metabolism in Drug Safety Evaluation

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7.1 SUMMARY

Over the past two decades, drug metabolism has become an indispensable component of both research and development activities in the pharmaceutical industry, where it has played an increasingly important role in support of the safety assessment of new chemical entities (NCEs). In response to recent regulatory guidance on the safety evaluation of metabolites of candidate drugs in man, novel approaches have been adopted to define the identities and levels of circulating metabolites in the plasma of both human subjects and the animal species employed in safety assessment programs. Through these analyses, exposure margins are established for human drug metabolites that circulate above a certain threshold in order to provide assurance that the preclinical toxicology program takes into account metabolites of the drug candidate as well as the parent molecule. Metabolites judged to be "disproportionate" or "unique" by regulatory criteria may require more detailed evaluation. In considering the factors that need to be taken into account in assessing the safety of metabolites of NCEs, it is appropriate to classify biotransformation products into two categories based on their chemical reactivity, viz. "stable" and "chemically reactive." The different experimental approaches employed to study these two groups are outlined and the implications of the findings for lead optimization efforts in drug discovery, and for later phase clinical development programs, are discussed in the context of establishing the human safety of the NCE.

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7.2 INTRODUCTION

Traditionally, the primary focus of safety assessment paradigms in pharmaceutical development centered on measuring exposure to the drug candidate itself, first preclinically in animal models to support first-in-human studies and, subsequently, during clinical trials to establish appropriate safety margins for continued clinical development. However, with the advances that have occurred over the past two decades in the field of drug metabolism [1], notably in bioanalytical technologies for the detection, identification and quantitative analysis of drug metabolites, and in the tools of molecular biology that have enabled studies on the biological function and regulation of drug metabolizing enzymes and transporter proteins, increased attention has been paid to the pharmacological and toxicological importance of drug *metabolites* in the drug discovery and development process. Indeed, the level of scrutiny of biotransformation products has been heightened by the growing awareness that the adverse effects of many foreign compounds may be attributed, at least in part, to the actions of one or more metabolites of the administered agent, and it is in this context that drug metabolism as a discipline has become an integral component of the safety evaluation of NCEs [2]. Regulatory guidance documents now have been published on the safety assessment of drug metabolites [3,4], with an emphasis on those biotransformation products that circulate (or are anticipated to circulate) in human plasma, the overall goal of which is to provide assurance that preclinical safety findings are relevant to the human situation in terms of exposure to both parent drug *and* its circulating metabolites. For purposes of discussion, drug metabolites may be divided into two broad categories, namely, those that are sufficiently stable to be isolated from biological fluids and characterized by conventional analytical techniques [e.g., liquid chromatography−tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance (NMR)], and those that are chemically reactive in nature and rarely are detected as the native species, but whose existence may be inferred from stable downstream products of further biotransformation [e.g., conjugates with glutathione, γ-glutamylcysteinylglycine (GSH)]. In general, these two groups of metabolites act to cause toxicity by different mechanisms, distinct approaches are employed to study them, and the implications for drug development associated with stable versus reactive metabolites also differ. At the heart of the matter in both cases, however, is the question of predicting drug toxicity in humans from results obtained in animals, where species differences in drug metabolism (which normally are quantitative, rather than qualitative) may confound the interpretation of animal toxicology data in terms of human risk assessment [5].

7.3 STABLE DRUG METABOLITES

The issue of addressing, in a systematic manner, the involvement of stable drug metabolites as a key component of the safety assessment of new drug candidates was first highlighted in a position paper coauthored by representatives of member companies of the US Pharmaceutical Research and Manufacturers Association (PhRMA), which proposed a number of practical approaches to the problem [6]. This publication, which became known as the *MIST* ("Metabolites in Safety Testing") document, proposed inter alia that those small molecule drug metabolites whose exposure in humans [as measured by area under the plasma concentration versus time curve (AUC_p) values]

exceeded 25% of that of the respective parent drug be viewed as "major metabolites" and that only such metabolites should be given further consideration in terms of verifying systemic exposure in one or more of the animal species employed in the preclinical safety assessment program. Recommendations also were made as to the specific, albeit very rare, case of a "unique" human metabolite, defined as a product of biotransformation that is detectable only in humans and not in any of the animal species investigated. In the years following publication of the MIST paper, numerous commentaries have appeared dealing with different aspects of the issue, such as whether absolute, as opposed to relative, metabolite exposure should trigger further safety evaluation of a given metabolite [7], the importance of the human radiolabeled absorption, distribution, metabolism, and excretion (ADME) study as the primary source of information on human metabolites for decision-making purposes [8], the impact of duration of drug administration on potential metabolite-mediated toxicity [9], and consideration of mechanisms of toxicity with regard to parameters such as dose, abundance, and duration of therapy [10]. Clearly, the challenge of addressing, in a comprehensive fashion, the safety of human drug metabolites is a complex and multifaceted issue.

In 2008, the US Food and Drug Administration (FDA) published a guidance document entitled "Safety Testing of Drug Metabolites" [3], which outlined the Agency's views on best practices in this area. In some respects, this guidance paralleled the recommendations of the MIST paper [6], but differed significantly in other respects, notably in defining the threshold for human metabolites of interest as those whose AUC_p values exceeded 10% of that of the parent drug when measured under steady-state dosing conditions. Moreover, it was recommended that metabolites falling into this category should be demonstrated to be present in the plasma of at least one of the animal species selected for preclinical safety assessment at levels at least as great as those observed (or predicted) in human subjects at the highest clinical dose. Should this requirement for exposure margins (i.e., animal $AUC_p \geq$ human AUC_p) not be met, then the metabolite in question is designated "disproportionate," and additional safety studies may be required to assure its human safety. While in some cases, metabolite exposure in animals can be increased by administering progressively higher doses of the parent drug, this approach is not always successful because of a number of factors, for example, limited solubility in the dosing vehicle, saturation of drug absorption, and species differences in metabolic pathways. In these instances, the FDA guidance recommends that separate animal toxicology studies with the preformed (synthetic) metabolite should be considered. There appears to be general agreement that drug conjugates present in the systemic circulation do not, in general, pose a safety concern, although the FDA guidance singles out acyl glucuronides as being "toxic molecules," a curiously broad generalization. Finally, the guidance emphasizes the need for information on human drug metabolism to be acquired as early as possible in the drug development process so that issues related to disproportionate human metabolites may be recognized and addressed before the initiation of large-scale (phase III) clinical trials.

The recommendations of the FDA guidance on MIST have a number of significant implications for the pharmaceutical industry, and many companies have revised (or are reviewing) their approaches to assessing the role of drug metabolites in the safety evaluation of NCEs. Central to many of the concerns over the MIST guidance are practical limitations that pertain to the conduct of drug metabolism studies in support

of drug development [11,12]. For example, it is widely accepted that the "gold standard" approach to quantitative metabolic profiling in human subjects is the radiolabeled ADME study [8,13], which typically is not performed until a relatively late stage of development (usually following the phase IIA proof-of-concept trials) and involves the administration of a ^{14}C-labeled variant of the drug candidate as a single dose; thus, definitive information on the presence of a disproportionate metabolite may not be available early in the development process, and the data would not be obtained under steady-state conditions which require a multiple-dose design. Moreover, any approach to assessing the toxicological properties of a drug metabolite that involves administration of the preformed compound is fraught with difficulties because of potential differences in the disposition of that metabolite when dosed as the synthetic material versus when the metabolite is formed *in vivo* from the parent drug [14,15]. Indeed, chemical synthesis of the metabolite of interest on a scale appropriate for *in vivo* studies may not be straightforward, particularly when issues of stereochemistry are taken into account, such that considerable time, effort, and financial resources may need to be expended for questionable return. Also, the 10% "cut-off" for a metabolite-of-interest seems unduly stringent, given that many situations are known in which the parent drug is a quantitatively minor contributor to the total drug-related material in human plasma due to extensive first-pass metabolism; in such cases, numerous circulating metabolites could exceed the 10% threshold and, if not present at exposure multiples greater than $1\times$ in animals would require separate toxicology testing. Clearly, the resource implications associated with addressing these issues are nontrivial, and the scientific value of conducting such studies in many cases may be marginal at best and misleading at worst.

Interestingly, the International Conferences on Harmonization (ICH) counterpart of the FDA MIST guidance, termed *document M3* [4], also adopts a 10% threshold for circulating drug metabolites but references this value to "total drug-related exposure" and not to parent drug alone. This is a significant difference between the two regulatory guidances, and begs the question of how best to determine "total drug-related exposure" early in the drug development process, before the conduct of the radiolabeled human ADME study. Also, ICH M3 makes no mention of metabolite assessment under steady-state conditions and also suggests that the above 10% figure may be relaxed somewhat for therapeutic agents that are to be administered at a daily dose of <10 mg. How these differences between the FDA and ICH guidances will be resolved remains to be seen, although it is anticipated that the ICH guidance will take precedence. Regardless, it is apparent that in the future drug metabolites will be the focus of much greater regulatory scrutiny from a drug safety perspective than has been the case in the past.

In response to the guidances dealing with the issue of drug metabolites in drug safety evaluation, numerous publications have appeared dealing with topics such as new (nonradioactive) methodologies for detecting, identifying, and quantifying metabolites from both animal studies and early (first-in-human) clinical trials, based on NMR spectroscopy [16,17] and LC-MS/MS with high resolution mass analysis [18–22]. An alternative approach that retains the use of radioactivity for early human metabolism studies is the "microdose" technique, in which a very low dose of ^{14}C-labeled drug (typically 100 μg or less and <1 μCi) is administered to volunteers in what may be regarded as a "pilot" human ADME study, and metabolite profiles in plasma determined by the combined use of (off-line) liquid chromatography (LC) and accelerator mass spectrometry (AMS) [23]. Since both the mass of drug candidate and the dose

of radioactivity are extremely low, such "Phase 0" human studies can be supported by a much-abbreviated preclinical safety package, thereby economizing on time and resources. However, this approach suffers from the limitation that, while radiochromatographic metabolite profiles can be reconstructed for the drug candidate of interest, no structural information is available from AMS, and therefore, additional work is required to identify any human metabolites that do not coelute on LC with previously characterized biotransformation products formed in animals or generated using *in vitro* systems. A related topic centers on our ability to predict circulating human metabolites from *in vitro* data, and an analysis of 17 molecules from the Lilly portfolio showed that in only 41% of cases were the plasma metabolite profiles adequately predicted from *in vitro* data [24]. A somewhat more favorable outcome was reported in a similar study by the Pfizer group [25], in which the success rate in predicting human excretory and circulating metabolite profiles from a panel of 48 compounds was judged to be $>70\%$ based on *in vitro* data obtained from pooled human liver microsomes, liver S-9 fraction, and/or human hepatocytes. However, it was noted that the predictability of secondary metabolites was less reliable than that with primary products of biotransformation, and the authors concluded that *in vitro* systems alone cannot mitigate fully the risk of disproportionate circulating metabolites in humans.

While the recommendations contained in the regulatory guidances on MIST likely will remain controversial for some time to come, it is noteworthy that many pharmaceutical companies, for example, Bristol–Myers Squibb [26,27], Lilly [28], and Pfizer [29], have published thoughtful analyses of the topic and, in some cases, have put in place formal strategies for the conduct of drug metabolism studies in support of drug safety evaluation that take into account the issues of disproportionate or unique human drug metabolites. There appears to be general agreement that a "flexible, tiered approach" should be adopted for the characterization and quantitative analysis of stable drug metabolites and that decisions on the need for safety studies on specific human metabolites be based on sound science and well-balanced judgment. Commentaries on MIST from the regulatory bodies themselves would appear to echo this sentiment [30–32].

Underlying many of the criticisms of the MIST guidances discussed above is the fact that relatively few examples exist where a stable drug metabolite has been clearly implicated as the causative agent in the adverse effects observed on dosing with the parent drug. This observation has been rationalized in terms of the normally modest structural changes that accompany mammalian biotransformation reactions, which suggests that most drug metabolites will exhibit off-target effects that are qualitatively similar to those of their respective parents, although quantitative differences can be expected because of changes in certain physicochemical properties, for example, polarity, which influences plasma protein binding and tissue penetration [12,26]. However, exceptions do exist, such as the neurotoxin 1-methyl-4-phenyl-pyridinium (MPP^+), formed by monoamine oxidase-B (MAO-B)-catalyzed metabolism of the illicit drug impurity 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [33], the acyl glucuronide conjugate of gemfibrozil, which serves as a mechanism-based inhibitor of CYP2C8 and thereby causes a number of drug interactions [34], and the sulfate conjugate of troglitazone, which has been shown to inhibit the hepatic transport proteins BSEP and OATP and is believed to contribute to cholestatic liver injury in diabetic patients treated with troglitazone [35] (Fig. 7.1). In these examples, the toxicity observed following administration of the respective parent is believed to be due to the stable metabolite

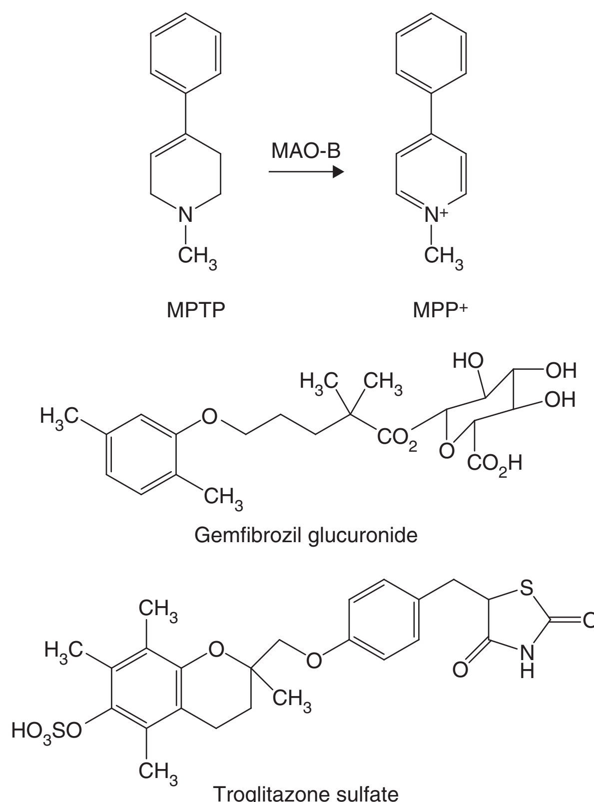


Figure 7.1 Structures of metabolites believed to be responsible for the adverse effects of their respective parent compounds. MPP^+ is formed as a product of MAO-B-mediated oxidative metabolism, while the acyl glucuronide and sulfate conjugates of gemfibrozil and troglitazone result from the action of UDP glucuronosyltransferase and sulfotransferase enzymes, respectively.

in question. Of course, in certain instances, biotransformation of a drug molecule will result in the formation of a stable metabolite whose structure differs markedly from that of the parent, as may occur on an internal cleavage mediated by oxidative or hydrolytic processes, and the toxicological properties of that metabolite may well be quite distinct from those of the parent, for example, cyanide ion formed on hydrolysis of the naturally occurring glycoside amygdalin (Leatrile) [36]. The reverse situation also can apply, namely, that the toxicity resides in the parent drug but metabolism leads to a nontoxic product because of a significant change in structure–activity relationships (SARs) for the biological targets that mediate the therapeutic effects versus the toxicity. A classic example of the latter scenario is found with terfenadine (Seldane), a potent histamine H1 antagonist that was marketed for the treatment of seasonal allergies. Although not appreciated at the time of launch in 1985, terfenadine binds avidly to human ether-à-go-go related gene (hERG) (the α-subunit of the cardiac IK_r (potassium-dependent delayed rectifier current) potassium channel), blockade of which leads to delayed ventricular polarization, prolongation of the QTc interval and, in extreme cases, potentially fatal cardiac arrhythmias [37]. In contrast, fexofenadine, the major metabolite of terfenadine in humans and the most abundant drug-related material

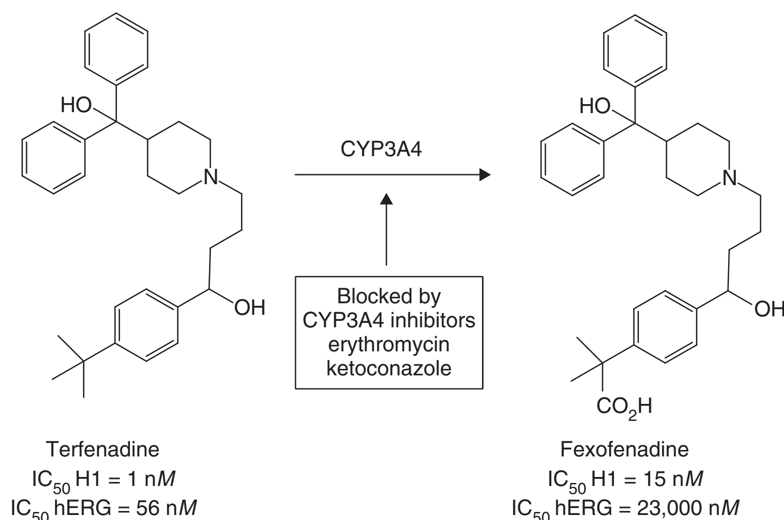


Figure 7.2 Metabolism of terfenadine to fexofenadine catalyzed by CYP3A4. This oxidation pathway results in a modest loss of activity at the histamine H1 receptor but in a large decrease in affinity for the hERG channel. Potent inhibitors of CYP3A4, for example, erythromycin and ketoconazole, effectively block the conversion of terfenadine to fexofenadine, and thereby increase exposure to terfenadine to levels that cause cardiotoxicity through prolongation of the QTc interval.

in the systemic circulation following an oral dose, has negligible binding affinity to hERG but retains good antihistamine activity. The conversion of terfenadine to fexofenadine in humans is catalyzed by CYP3A4 [38], and potent inhibitors of this enzyme, such as erythromycin and ketoconazole, block the metabolic "detoxification" process and lead to blood levels of terfenadine at which cardiotoxicity is manifest (Fig. 7.2). Recognition of the molecular basis of this serious drug–drug interaction led to the removal of terfenadine from the market in 1997 and its replacement by the metabolite fexofenadine (Allegra). While this drug interaction attracted widespread attention and had profound consequences for the pharmaceutical industry (introduction of high throughput screens for hERG binding in drug discovery programs), it also underscores the necessity to fully understand, at as early a stage as possible, the spectrum of off-target activities associated not only with the parent drug, but with its major circulating metabolites. This applies particularly to inhibition of the hERG channel (potential for cardiotoxicity) and to the inhibition of human CYP enzymes (potential for drug–drug interactions). While SARs have been relatively well established for hERG inhibition [39], much remains to be learned about those for CYP inhibition, and relatively few studies have examined the contribution of circulating human metabolites to drug–drug interactions that occur at the level of CYP inhibition [40].

7.4 REACTIVE DRUG METABOLITES

As pointed out above, there are relatively few cases where stable drug metabolites are believed to mediate the toxicity of their respective parent compounds. In contrast,

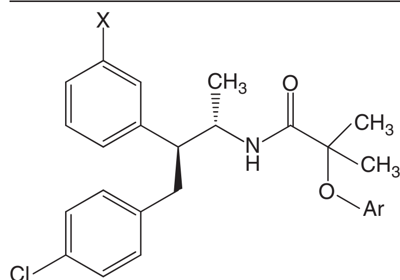
chemically reactive metabolites, most commonly generated via oxidative reactions catalyzed by the cytochrome P450 (CYP) family of enzymes [41], have been implicated in a variety of drug-induced toxicities [5,42,43]. The deleterious effects of these short-lived electrophilic species are thought to arise from their propensity to cause oxidative stress and/or to covalently modify biological macromolecules (primarily proteins and nucleic acids) and thereby alter normal cellular function. For example, covalent adduction of DNA by “hard” electrophiles can lead to mutagenesis, carcinogenesis, and teratogenesis [44], while “soft” electrophiles typically target nucleophilic centers on peptides and proteins and have been implicated in both target organ toxicities (notably those involving the liver [45–47]) and idiosyncratic reactions mediated by the immune system [48–51]. In the area of human pharmaceuticals, most reactive metabolites fall into the category of “soft” electrophiles, the formation of which can be inferred from their *S*-linked conjugates with the tripeptide GSH or through their covalent adducts with proteins which are most effectively detected with the aid of a radiolabeled analog of the drug [1,52]. It is of interest to note that, of the four broad categories of drug-induced toxicity proposed by Park *et al*. [53], reactive metabolites are proposed to play a central role in three of these mechanisms, when they have been associated mainly with hepatotoxicity, skin reactions, and blood dyscrasias [42]. However, it is equally important to point out that not all reactive metabolites are toxic, and the formation of adducts with GSH and proteins is not, *per se*, indicative of a hazardous compound [52]. Nevertheless, an analysis of drugs that have been removed from the market (or labeled with a “black box” warning) because of safety concerns has shown that, next to primary or secondary pharmacology, the major reason was direct organ toxicity, potentially associated with metabolic activation events. This suggests that reactive metabolite formation, while not an accurate predictor of drug toxicity in humans, represents a significant risk factor in drug development.

Growing concerns over the high attrition rates in drug development, and the recognition that toxicity is a major contributing factor, has led most pharmaceutical companies to implement strategies that aim to minimize potential metabolic activation liabilities in their development candidates. In 2004, the Merck group proposed a strategy based on an assessment of the covalent binding properties of lead compounds emerging from drug discovery programs, and outlined a set of decision-making criteria on which to advance lead candidates [54]. Metabolic profiling studies were performed in parallel to the measurements of covalent binding, focusing on the formation of GSH–drug conjugates whose structures provide a rationale for chemical modification to abrogate the formation of reactive metabolites [55]. Updates to the Merck strategy have been published as more practical experience has been gained [56–59], and a number of commentaries have appeared on approaches to minimize metabolic activation in drug discovery programs [60–63]. Much has been made of “toxicophores” or “structural alerts” (structural motifs that have been associated with metabolic activation to reactive electrophiles) [64], and Kalgutkar *et al*. [65] have published a comprehensive listing of functional groups that have been shown, in certain molecules, to undergo bioactivation to electrophilic intermediates. While helpful to the medicinal chemist engaged in drug design, it should be borne in mind that the presence of a toxicophore in a given drug structure is relevant only when that functional group is accessible to the appropriate activating enzyme in a biological milieu; in that context, it is important to assess experimentally whether metabolism of a promising drug candidate indeed occurs at the functionality of concern before rejecting the molecule from consideration. In some

examples, the toxicophore may be as simple as an electron-rich benzene ring, as was the case in the lead candidate in a cannabinoid-1 receptor (CB-1 R) inverse agonist program (compound **1**, Table 7.1), which underwent sequential metabolic oxidations to afford reactive arene oxide and *ortho*-quinone intermediates that were captured in the form of their GSH adducts [66,67]. Labeling of **1** with tritium demonstrated that this compound gave rise to very high levels of protein covalent binding (~3900 pmol-equiv/mg protein) in both rat and human liver microsomal preparations, and a series of analogs were prepared with the goal of reducing this bioactivation liability, while simultaneously retaining (or enhancing) the favorable pharmacological and pharmacokinetic characteristics of the lead structure. The modifications centered on reducing the electron density on the offending aromatic ring, through introduction of fluorine atoms (**2**), replacement of the phenoxy substituent by a 2-pyridyloxy (**3**) or 5-CF_3-2-pyridyloxy group (**4**), each of which led to progressively lower levels of covalent binding *in vitro*. The final compound in the series (**5**), which incorporated a nitrile substituent in the distal phenyl ring, exhibited a very low level of covalent binding (27 pmol-equiv/mg protein), had excellent Pharmacokinetic (PK) properties in rats and was almost an order of magnitude more potent at the CB-1 R than the initial lead. On the basis of these attributes, compound **5** was advanced into development, and became Taranabant, Merck's candidate CB-1 R inverse agonist for the treatment of metabolic syndrome [68].

In those situations, where electrophilic metabolites are highly reactive, alkylation of the apoprotein and/or prosthetic heme moiety of the responsible CYP enzyme may occur, resulting in mechanism-based inactivation of the enzyme. This phenomenon, which manifests as a time-dependent loss of enzyme activity *in vitro* (and often is the first indication that a new drug candidate is subject to metabolic activation), has potentially important safety implications for clinical drug–drug interactions with cosubstrates of the P450 isoform in question, and molecules with this undesirable property normally are "screened out" at the discovery stage [69–72]. Similarly, metabolism of certain functional groups, notably the methylenedioxyphenyl moiety and substituted aliphatic amines, affords electron-deficient intermediates that coordinate tightly to the Fe^{II} oxidation state of CYP heme and form inhibitory "metabolic intermediate" (MI) complexes that can be detected spectrophotometrically through their characteristic λ_{max} at 455 nm [71,72]. An example of a lead optimization program which focused on the removal of MI complex formation from an important lead molecule was reported by Tang *et al*. [73] and illustrates how an early understanding of potential metabolic liabilities in a given structural class can be key in guiding medicinal chemistry efforts. *In vitro* investigation of the melanocortin-4 receptor (MC4R) agonist **6** (Fig. 7.3), being explored for a weight-loss indication, showed that this compound inhibited CYP3A enzyme activity in nicotinamide adenine dinucleotide phosphate (NADPH)-fortified rat and human liver microsomal preparations and yielded a typical MI complex spectrum when incubated with recombinant CYP3A4 (Fig. 7.4). Since compound **6** contained an aliphatic amine moiety, it was hypothesized that sequential CYP-mediated N-oxidation afforded the corresponding hydroxylamine and nitroso derivatives and that the latter species coordinated with P450 heme iron to generate the observed MI complex. This hypothesis was tested by *in vitro* trapping experiments with cyanide ion [74], which resulted in the identification by LC-MS/MS of a cyano adduct whose formation could be rationalized by invoking capture, not of the nitroso species per se, but of the tautomeric oxime, followed by a further two-electron oxidation step of the adduct to give the final

TABLE 7.1 Covalent Binding and Rat Pharmacokinetics of Investigational CB-1 R Inverse Agonists



Compound	Ar	X	CB1R IC_{50} (nM)	Covalent Binding (pmol-equiv/mg)	Rat Pharmacokinetics[a]			
					CL_p [(mL/min)/kg]	$t_{1/2}$ (h)	C_{max} (μM)	F (%)
1	Ph	H	2.03	3900	33	2.8	0.18	9
2	3,5-F_2-Ph	H	1.47	1700	35	2.4	0.30	19
3	2-pyr	H	1.80	910	25	2.2	0.44	29
4	5-CF_3-pyr	H	0.54	88	35	2.2	1.2	100
5	5-CF_3-pyr	CN	0.29	27	33	2.7	0.49	74

[a] CL_p, rate of compound clearance from plasma; $t_{1/2}$, half-life of compound circulating in plasma; C_{max}, maximum concentration of compound achieved after an oral dose of 2 mg/kg; F, oral bioavailability.
Source: Adapted from Ref. 67.

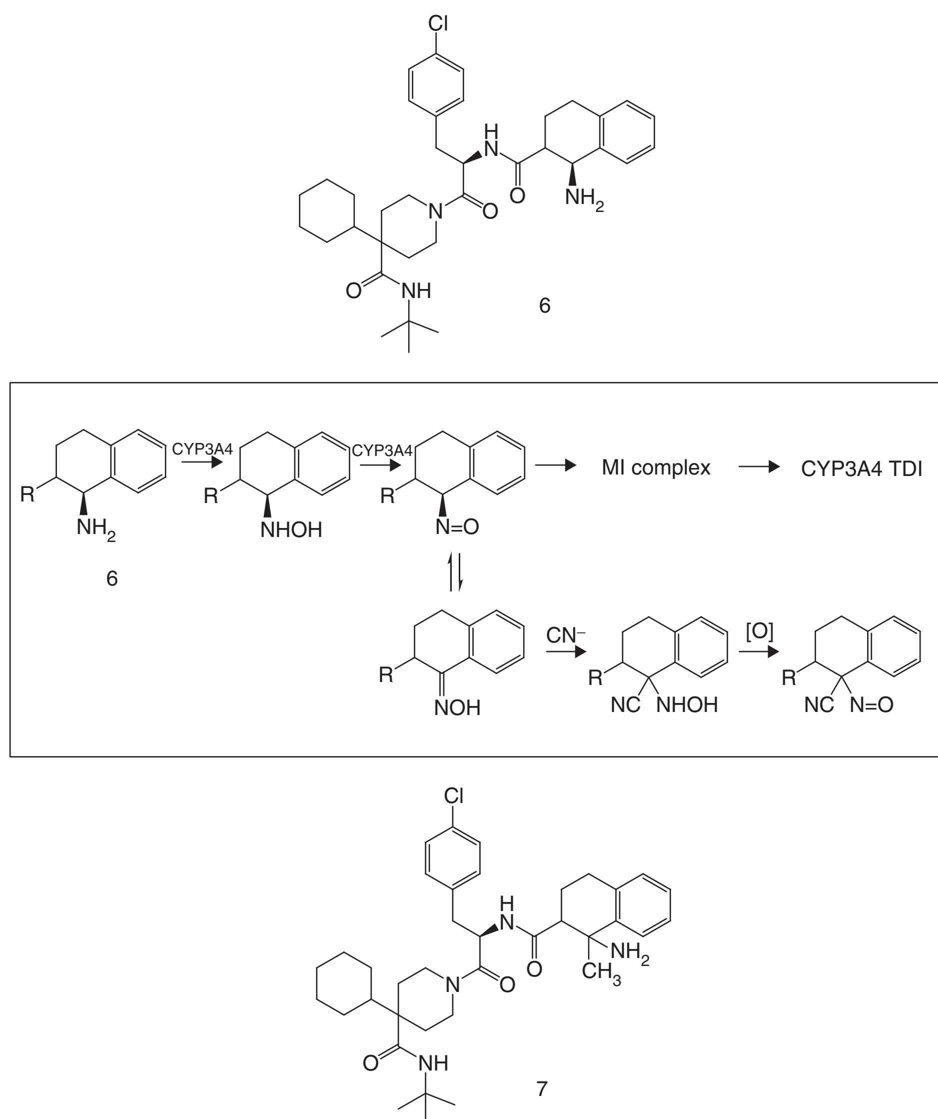


Figure 7.3 Structure of a lead MC4R agonist **6** and the proposed mechanism by which it caused time-dependent inhibition (TDI) of CYP3A4. Trapping experiments with CN^-, which afforded the indicated cyano-nitroso species, led to design of **7**, in which the methyl substituent α- to the primary amine introduced sufficient steric hindrance to suppress oxidation at this center to the inhibitory nitroso metabolite.

product (Fig. 7.3). These findings led to the design of compound **7**, in which a methyl group was introduced α- to the primary amine functionality in order to sterically hinder the offending N-oxidation reaction. This simple structural modification afforded a compound with greatly diminished MI complex formation and CYP3A inhibitory properties, thereby provided a drug candidate with an improved safety profile in terms of potential drug–drug interaction liabilities [73].

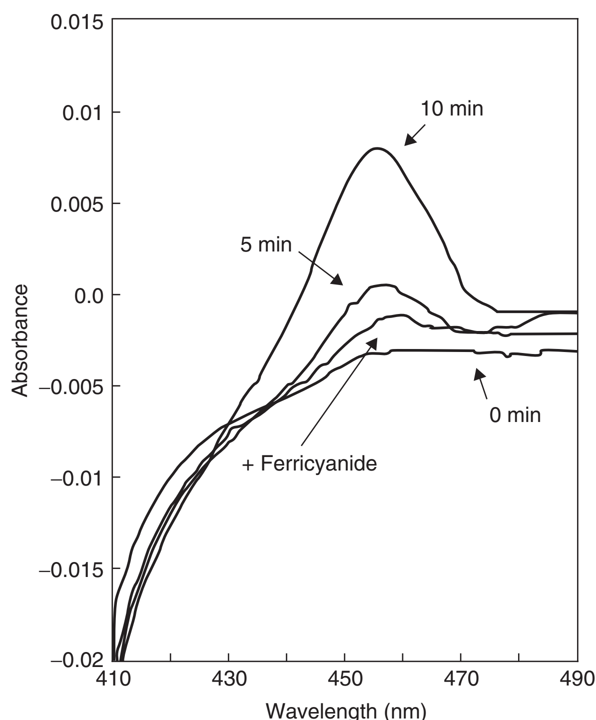


Figure 7.4 Spectrophotometric detection of the MI complex formed in incubations of **6** with recombinant CYP3A4. The absorbance increased with increasing incubation time (0, 5, and 10 min) and decreased on addition of potassium ferricyanide which converts heme iron to the Fe^{III} oxidation state and dissociates the MI complex. *Source*: Adapted from Ref. 73.

In the foregoing examples, it will be evident that a number of analytical techniques are employed in the detection of reactive metabolite formation, for example, UV spectroscopy for MI complexes, use of radiotracers for metabolic profiling and covalent binding studies, and *in vitro* nucleophilic trapping experiments with structural analysis by LC-MS/MS or NMR. By far, the most widely used nucleophile for the latter purpose is GSH, due both to its physiological role as an endogenous scavenger of potentially damaging electrophiles and to the favorable mass spectrometric characteristics of the resulting drug–GSH adducts under a variety of ionization conditions [75–78]. In recent years, a variety of LC-MS/MS-based methods have been reported for screening drug candidates for reactive metabolite formation *in vitro*, based on trapping experiments with GSH itself, or analogs such as GSH ethyl ester [79], quaternary ammonium derivatives of GSH [80], and stable isotope-labeled ($^{13}C_2$ and ^{15}N) GSH [81,82]. An interesting variation on this theme is the use of γ-glutamylcysteinyllysine (γ-GSK) as a bifunctional trapping agent, which has been shown to capture both “soft” and “hard” electrophiles [83]. The advent of LC-MS/MS systems with rapid scanning mass analyzers (e.g., time-of-flight) capable of operating under conditions of high mass resolution has provided an additional dimension in screening technology, based on elemental composition data and the technique of mass defect filtering [82,84–87]. Despite the power of mass spectrometry-based methods for the detection

and identification of GSH adducts, such approaches do not yield reliable information of a quantitative nature in the absence of authentic standards of the conjugates of interest because of differences (sometimes quite marked) in the ionization efficiency of individual adducts. Attempts have been made to address this problem by the use of ^{3}H- or ^{35}S-labeled GSH as trapping agents, with radiometric analysis of the products [88,89] or dansyl GSH followed by fluorometric analysis [90], both of which can provide quantitative information in the absence of synthetic reference materials. It is generally assumed that the reaction of GSH with reactive drug metabolites *in vitro* is predominantly a chemical process, although in certain cases, glutathione-*S*-transferase-mediated conjugation may contribute to product formation; analogs of GSH (or GSH surrogates, e.g., *N*-acetylcysteine) would not be appropriate for quantitative purposes in such situations since these nucleophiles do not serve as substrates for the transferase enzymes. While efforts have been made by some investigators (with limited success) to correlate thiol formation in liver preparations *in vitro* with measures of covalent binding *in vivo* and hepatotoxicity in animals or humans [91,92], it would seem that the greater value of GSH trapping experiments lies in the structural information that can be inferred on the identities of the reactive electrophiles being captured. When this insight is provided at a sufficiently early stage in drug discovery programs, rational structural changes can be made in lead compounds to block, or suppress, pathways of bioactivation, thereby minimizing the potential for reactive metabolite-mediated toxicities [93]. Although the foregoing discussion has focused on the detection of reactive metabolites using appropriate *in vitro* systems, extension of these studies to *in vivo* investigations typically rely on the collection of bile from animals dosed with the NCE of interest, and the identification of GSH adducts present in that fluid. Alternatively, the excretion of *N*-acetylcysteine (mercapturic acid) conjugates in urine, either of animals or human subjects, provides an insight into bioactivation processes occurring *in vivo* [77].

In assessing the potential risk to humans associated with a development candidate that has been demonstrated to undergo metabolism to reactive intermediates, a number of considerations need to be borne in mind, for example, the chemical tractability of the structural series from which the candidate was selected, the availability of existing treatments for the target disease, the severity of that disease (life-threatening or otherwise), the anticipated daily dose (and hence body burden of reactive metabolites), the expected duration of therapy (which will determine long-term exposure to reactive metabolites), the intended clinical population (adult vs children), and the balance between bioactivation and detoxification pathways *in vivo* [54,93]. It also should be recognized that chemically reactive intermediates will exhibit a large range of reactivities in a biological environment, which will influence their ability to migrate from sites of formation within the cell to other organelles, or even to distant organs. For example, the diquinonemethide metabolite of raloxifene, a selective estrogen receptor modulator, has been shown to have a half-life of <1 s in aqueous phosphate buffer at 5°C, while an *ortho*-quinone metabolite of the same drug exhibited a half-life of 69 min in the same medium at 37°C [94] (Fig. 7.5). Thus, while the majority of electrophilic drug metabolites have biological lifetimes that are too short to allow for their detection and characterization as the native species, a subset of these products will have relatively low chemical reactivities and therefore will be detectable in the circulation and excreta. A prominent example of the latter group is the class of acyl glucuronide conjugates, which are formed from many carboxylic acid-containing xenobiotics and frequently

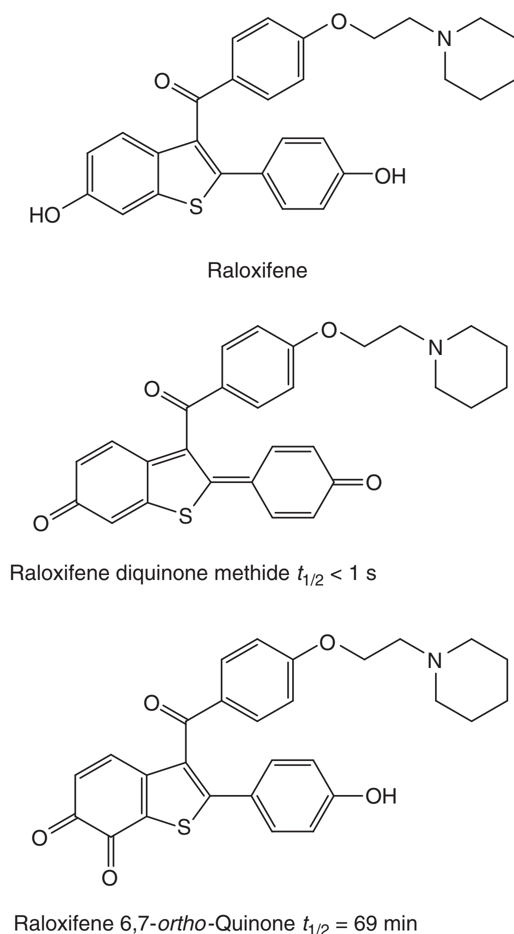


Figure 7.5 Structures of raloxifene and two quinoid metabolites with markedly different stabilities in aqueous phosphate buffer.

circulate in the bloodstream or are eliminated in bile or urine [95–98]. Owing to the propensity of these ester-linked conjugates to undergo intramolecular rearrangement reactions with resulting exposure of α-hydroxy-aldehydes from the sugar ring, they possess the ability to form stable covalent adducts to proteins. Direct acylation of proteins also can occur with acyl glucuronides (as is the case with the much more reactive acyl-coenzyme A thioesters, which usually do not escape the cell). In light of their ability to covalently modify proteins, concerns have been raised over the safety of acyl glucuronides as a structural class. Specifically, it has been noted that acyl glucuronide conjugates may be concentrated in hepatocytes and the biliary tree due to vectorial transport by export pumps, where they may bind selectively to canalicular membrane proteins [95]. Such concerns have been heightened by the fact that a relatively large proportion of drug withdrawals because of hepatotoxicity have involved carboxylic acid derivatives that yield acyl glucuronide conjugates [99]. Despite the fact that more recent investigations of some of these agents (e.g., zomepirac and tolmetin [100]) have

revealed pathways of bioactivation that involve metabolic *oxidations*, there has been a tendency to associate acyl glucuronide formation with a risk of adverse drug reactions, notably liver injury. Such a broad generalization seems inappropriate, since many carboxylic-acid-containing therapeutic agents that give rise to acyl glucuronides have a favorable safety profile, yet acyl glucuronide conjugates are referred to collectively in the FDA guidance as *toxic molecules* [3]. While it may be possible to assess the intrinsic reactivity of a particular acyl glucuronide *in vitro*, for example, by monitoring rates of intramolecular migration or direct acylation of model nucleophiles, there currently is a lack of consensus on the value of such information for risk assessment purposes. It is perhaps more appropriate, therefore, to treat this class of conjugates in the same fashion as stable drug metabolites, where exposure margins in animal species are taken as indices of safety in humans.

Unlike the situation with stable drug metabolites, there are no regulatory guidances that deal specifically with chemically reactive metabolites, although the European Medicines Agency acknowledges that drug-induced hepatotoxicity may, in some cases, be mediated by reactive metabolites [101]. The lack of a direct correlation between the formation of electrophilic drug metabolites and drug-induced toxicity has confounded the interpretation of data on metabolic activation and covalent binding to proteins and underscores our general lack of understanding of the molecular mechanisms by which reactive intermediates damage cells [52]. While studies aimed at correlating the extent of covalent binding *in vitro* of hepatotoxic versus nonhepatotoxic drugs have yielded conflicting results [102,103], experimental protocols based on the use of human hepatocytes that incorporate estimates of dose (or body burden of reactive metabolites) have shown some promise as predictors of hepatotoxic potential [92,104–106]. It has been suggested that, for practical purposes, an estimated reactive metabolite body burden in humans of $<$10 mg/day may represent an acceptable risk [7,8], although this proposed upper limit probably will be highly dependent on the nature of the reactive metabolite in question, the identities of its macromolecular targets, and individual host susceptibility factors. Currently, very little is known about the protein targets of reactive drug metabolites and even less about those structural modifications induced by protein alkylation that have toxicological consequences [107]. Advances in the techniques of proteomics mass spectrometry have made it possible to address the structural issues [108], but analysis of the hundreds (or thousands) of proteins that are subject to chemical modification by reactive drug metabolites presents a formidable challenge. For these reasons, it seems prudent, in the context of drug discovery programs, to incorporate a formal assessment of metabolic activation of NCEs at an early stage (e.g., during lead optimization) with a view to minimizing, through appropriate structural modifications, the risk of downstream adverse drug reactions mediated by chemically reactive metabolites. As noted above, most major pharmaceutical companies now subscribe to this general approach, although individual corporate strategies differ in terms of the timing of such studies and their experimental design.

In considering the role of reactive intermediates in drug toxicity, it should be pointed out that some important therapeutic agents actually elicit their pharmacological effects through chemically reactive metabolites or products of chemical rearrangement that covalently modify their biological targets. Thus, the thieno-tetrahydropyridine antiplatelet agents (e.g., ticlopidine and clopidogrel [109]) and the proton pump inhibitors (e.g., omeprazole, esomeprazole, and lansoprazole [110]) undergo conversion *in vivo* to reactive sulfenic acid species which, in turn, form disulfide

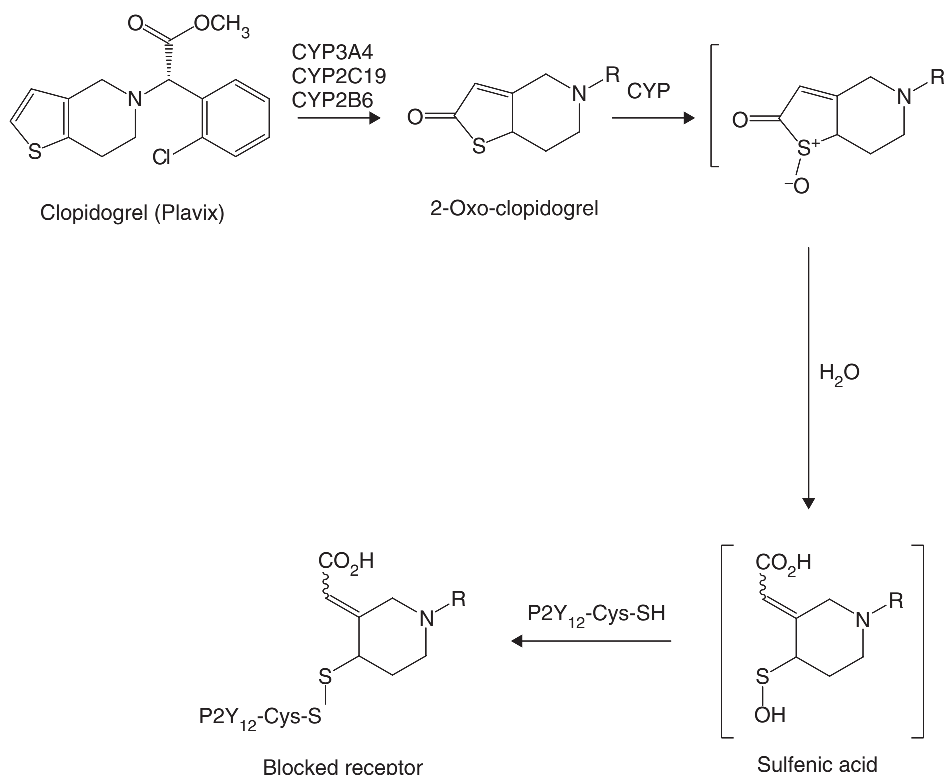


Figure 7.6 Cytochrome P450-mediated metabolism of clopidogrel to an acyclic sulfenic acid that binds covalently to an active site cysteine -SH group on the platelet $P2Y_{12}$ receptor.

linkages with active site -SH groups of their respective targets ($P2Y_{12}$ receptor on the surface of blood platelets and H^+/K^+ adenosine triphosphatase (ATPase) at the secretory surface of the gastric parietal cell). As an example, the mechanism by which clopidogrel (Plavix) inactivates its target receptor is depicted in Fig. 7.6. The relative safety of these widely used drugs probably is related to the high degree of specificity with which their active moieties interact with their target proteins, and the fact that the covalent linkage in each case is a disulfide bond, which can be reduced over time to regenerate the native protein. Interestingly, this concept of specific, reversible covalent modification of proteins has been advanced as a discrete strategy in the design of a new generation of potent, relatively long-acting therapeutic agents with low potential for toxicity [111].

Finally, reactive metabolites need to be taken into consideration when assessing the genetic safety of new pharmaceuticals, given the well-established role of drug metabolism in the genotoxicity and carcinogenicity of chemicals [44]. Traditionally, the potential of a new drug candidate and its mammalian metabolites to cause genetic toxicity is evaluated during the early stages of drug development through a combination of *in vitro* studies (e.g., using liver microsomal preparations from Aroclor-pretreated rats to enhance the production of metabolites) and *in vivo* assays (e.g., micronucleus assay in rat or mouse) [2]. Only in those cases where metabolism studies have identified a "unique" human metabolite, or where the structure of a metabolite of the

drug candidate of interest contains a genotoxicity “alert,” are follow-up studies normally performed with the metabolite per se. For example, when a human metabolite is not generated in rats or mice, assessment of its genotoxic potential could include the use of an alternative or optimized *in vitro* metabolic activation system or direct testing of the isolated or synthesized metabolite [112]. In recent years, a number of *in silico* approaches, involving expert systems or statistical modeling techniques, have been applied to predict human genotoxicity risk [113], and these have been adopted by both pharmaceutical companies and regulatory agencies as a screen for potentially problematic drug candidates, their known metabolites, and synthetic impurities in the active pharmaceutical ingredient. In situations where a drug candidate yields a human metabolite that tests positive in one or more assay in a genotoxicity panel, due consideration needs to be given to issues such as the intended therapeutic indication of the parent compound, the projected dose and duration of treatment, and the likely body burden of the genotoxic intermediate, in developing an overall strategy for risk assessment [114].

7.5 CONCLUSIONS

On the basis of the above discussion and the examples presented in this chapter, it will be evident that drug metabolism as a discipline plays a unique role within the pharmaceutical industry, in that it serves to bridge drug discovery projects to both preclinical and clinical drug development. In particular, a sound knowledge of the pharmacokinetic, metabolic, and dispositional characteristics of NCEs in animals, coupled with information on known or suspected human metabolites, represents a critical body of data for validating the choice of the animal species selected for the preclinical toxicology program. Evidence for the formation of chemically reactive metabolites has clear implications for the medicinal chemist with regard to logical directions for lead optimization, while at the clinical level, information on exposure margins for circulating stable metabolites dictates the need, if any, for further safety evaluation of specific biotransformation products. For all these reasons, drug metabolism studies represent an integral component of today’s drug safety assessment programs.

Looking to the future, one can anticipate that current advances in the fields of genomics, proteomics, and metabolomics will combine to provide a detailed insight into the numerous mechanisms by which foreign compounds elicit their specific adverse effects, and thereby provide a link between chemical structure and molecular toxicology. New tools will emerge at the drug metabolism/safety assessment interface, for example, the use of genetically engineered mouse models that express human P450 enzymes to address issues related to species differences in metabolism and toxicity [115,116]. An improved understanding of the role of redox-sensitive signaling pathways, for example, Kelch-like ECH-associated protein 1 (Keap1)-Nrf2-ARE [117], in protecting against the potentially deleterious effects of reactive drug metabolites, could lead to the development of *in vitro* screens for chemical stress and idiosyncratic drug toxicity, and improved biomarkers of cellular damage due to exposure to electrophilic drug metabolites likely will emerge, for example, induction of heme oxygenase-1 in human hepatocytes [118]. The intriguing question of what makes one human subject more susceptible to the toxic effects of a drug/drug metabolite than hundreds (or thousands) of other patients may be answered, at least in part, by genetic determinants

in the immune system, as has been shown to be the case with abacavir hypersensitivity which is strongly correlated with the presence of the human leukocyte antigen (HLA)-B*5701 [119]. It seems inevitable that the Holy Grail of predictive toxicology will emerge from close partnerships between drug metabolism and industrial safety assessment—an exciting prospect indeed!

ABBREVIATIONS

ADME	absorption, distribution, metabolism, and excretion
AMS	accelerator mass spectrometry
ARE	antioxidant response element
ATPase	adenosine triphosphatase
AUC_p	area under the plasma concentration versus time curve
CB-1 R	cannabinoid-1 receptor
CYP	cytochrome P450
FDA	the US Food and Drug Administration
GSH	glutathione, γ-glutamylcysteinylglycine
hERG	human ether-à-go-go related gene
ICH	International Conferences on Harmonization
IK_r	potassium-dependent delayed rectifier current
Keap1	Kelch-like ECH-associated protein 1
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MAO-B	monoamine oxidase-B
MC4R	melanocortin-4 receptor
MI	complex metabolic intermediate complex
MIST	metabolites in safety testing
NADPH	nicotinamide adenine dinucleotide phosphate
NCE	new chemical entity
NMR	nuclear magnetic resonance spectroscopy
SAR	structure–activity relationship
TDI	time-dependent inhibition
Nrf-2	nuclear factor-erythroid 2-related factor 2

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